VOL. 106 NOS. 2–3 JUNE 1st 1999 ISSN: 0378-4274
TOLED5 106 (2–3) 89–262 (1999)

Toxicology Letters

Toxicology Letters

Aims and Scope

Toxicology Letters serves as a multidisciplinary forum for research in all areas of toxicology. The prime aim is rapid publication of research letters with sufficient importance, novelty and breadth of interest. In addition to research letters, papers presenting hypotheses and commentaries addressing current issues of immediate interest to other investigators are invited. Mini-reviews in various areas of toxicology will also be published. A new feature is the provision of a forum for the discussion and interpretation of data published in the journal. Clinical, occupational and safety evaluation, legal, risk and hazard assessment, impact on man and environment studies of sufficient novelty to warrant rapid publication will be considered.

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Publication information: *Toxicology Letters* (ISSN 0378-4274). For 1999 volumes 104–111 are scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where Air delivery via SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues should be made within six months of our publication (mailing) date.

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Toxicology Letters

An International Journal for the Rapid Publication of Short Reports on all Aspects of Toxicology Especially Mechanisms of Toxicity

Volume 106 (1999)

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Commentary

Mandates to develop non-mammalian models for chemical immunotoxicity evaluation: are fish a viable alternate to rodents?

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Received 21 September 1998; received in revised form 20 January 1999; accepted 21 January 1999

Immunotoxicology, a relatively young focus area within the field of toxicology, began to achieve autonomy as an independent discipline in about the mid-1970s. A major concern of individuals working in this new area was accurately identifying immunotoxicants, and subsequently, understanding specific mechanisms by which these agents cause immunosuppression and/or exacerbate aberrant immune function (i.e. hypersensitivity, autoimmune disease). The realization that the immune system was a frequent target of toxic insult following chronic, subchronic, or acute exposure to environmental chemicals defined the need for development of immune assays suitable for use in screening potential immunotoxic compounds. Therefore, during the 1980s, the National Toxicology Program (NTP) sponsored a coordinated effort by several major laboratories to develop a testing paradigm which could reliably identify immunotoxic compounds (Luster *et al.*, 1988). The NTP-approved tier testing procedure (developed in B6C3F1 mice) proved to be effective but labor-intensive, thus, it became desirable to determine the simplest configuration of immune tests in the tiers that could reliably identify immunotoxicants. Data from over 50 compounds were compiled and used to generate estimates of the individual and pairwise ability of assays in the screening battery to detect immunotoxic compounds. It was found that the performance of only two or three select immune tests was sufficient to predict immunotoxic compounds in rodents ($>90\%$ concordance) (Luster *et al.*, 1992). Preliminary screening of compounds for immunotoxicity therefore often involves using a small number of immune tests with high predictive value for immunosuppression, rather than proceeding through numerous tests arranged in tiers.

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PII: S0378-4274(99)00018-1

Recently, the National Institutes of Health (NIH) received a mandate from Congress to provide increased support for development of non-mammalian models for toxicity testing (e.g. phylogenetically lower species, in vitro testing, computer models). We responded to an NIH Request for Applications (RFA) answering this mandate, by suggesting that we would recreate in a fish species the NTP immunotoxicity testing paradigm developed in mice, and in so-doing, determine if fish had efficacy as a non-mammalian model for detecting chemical immunotoxicants. Our reasoning was that fish (a) were inexpensive to produce and maintain, (b) possessed an immune system comparable in many ways to mammals (Wester *et al.*, 1994), and (c) appeared to be highly sensitive to immunotoxicant exposure. Regarding immunosuppression in fish, numerous studies using diverse fish species have demonstrated impaired immunity following environmental chemical exposure. Humoral immune function was inhibited in carp (*Cyprinus carpio*) exposed to lindane (Cossarini-Dunier *et al.*, 1987) or trichlorphon (Cossarini-Dunier *et al.*, 1990). Suppression of antibody production by rainbow trout (*Salmo gairdneri*) spleen cells following exposure to heavy metals (Anderson *et al.*, 1989) or the organochlorine 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Anderson *et al.*, 1983) has also been observed. Trace exposure to heavy metals also inhibits leukopoiesis in goldfish (*Carassius auratus*) (Murad and Houston, 1988) and phagocytic function in rainbow trout (*Salmo gairdneri*) (Elsasser et al., 1986). Recent reports indicate that impaired activity of phagocytic cells may be a sensitive indicator of environmental chemical exposure (polycyclic aromatic hydrocarbons and organotins) in wild fish. For instance, spot (*Leiostomus xanthrus*) and hogchoker (*Trinectes maculatus*) had depressed macrophage phagocytosis, chemotaxis, and chemiluminescence responses (Weeks and Warinner, 1986; Weeks et al., 1986, 1987, 1990). Further, the guppy (*Poecilia reticulata*) and medaka (*Oryzias latipes*) were both shown to develop immunopathological changes following exposure to methyl bromide and sodium bromide (Wester *et al.*, 1988). In our laboratory, previous experience has shown tilapia to be sensitive to immunotoxicants including PAH (Hart *et al.*, 1997), insecticides (Holladay *et al.*, 1996), and TCDD (Hart *et al.*, 1999).

Tilapia (*Oreochromis niloticus*) were selected as the research fish model because these fish were easily produced, economically important (Centers for Epidemiology and Animal Health, 1995), and could be grown large enough to provide sufficient immune cell numbers to conduct the desired immune assays. Fourteen chemical agents were selected to develop the tilapia immunotoxicity risk assessment model, consisting of nine well-established rodent immunotoxicants and five compounds presently defined as not immunotoxic to rodents. Immune tests to be conducted in fish included peripheral lymphocyte differentials, splenic white blood cell enumeration, lymphocyte blastogenesis (B and T cell mitogen assays and mixed-lymphocyte response), sheep RBC antigen-specific antibody production (using the plaque-forming cell assay, PFC), and antigen-specific cytotoxic T lymphocyte and natural killer (NK) cell cytolytic activity. Two NTP immune tests were not conducted in tilapia, specifically quantitation of lymphocytes using monoclonal antibodies (commercially unavailable) and the delayed-type hypersensitivity (DTH) response.

Results of the above testing indicated that tilapia were immunologically sensitive, typically showing inhibited immune responses at lower chemical exposure levels (mg/kg) than those used in the rodent model (data not shown). However, one rodent immunotoxicant, diethylstilbestrol (DES), did not inhibit the selected immune assays in fish (thus was identified as a non-immunotoxicant), and one of the five rodent non-immunotoxicants (*t*-butylhydroquinone) caused inhibition of at least one of the immune tests used in the fish. Antigen challenge assays (i.e. bacterial pathogens) were not included as endpoints in the study design, thus it is presently unknown if DES may actually be immunotoxic in tilapia, or similarly, if *t*-butylhydroquinone would produce positive results in challenge studies. To summarize, eight of nine rodent immunotoxicants were identified as immunotoxicants using the fish model (thus one 'false negative' was observed), while one of five rodent non-immunotoxicants appeared to pro-

duce immunotoxicity in tilapia (thus one apparent 'false positive').

Given that false negative results are probably of greater concern in a preliminary risk assessment study than are false positives, the correct identification of all rodent immunotoxic chemicals but one in this 'first-attempt' fish model may be seen as encouraging. With refinement of the fish model, it may be that an even closer parallel to rodent results could be obtained. However, numerous challenges were encountered when developing and implementing the present tilapia risk assessment model which need to be considered. Throughout these studies, high variability in data was a consistent problem. The extent of this problem was not totally appreciated at the onset, given that the tilapia used represent a somewhat inbred (but not syngeneic) population. It appeared that the fish were more genetically diverse than anticipated, and thus, large sample sizes were required. Another problematic area concerned the fish spleen, which proved to be an unsatisfactory source of lymphocytes for many of the planned immune assays. Specifically, the mature tilapia spleen contained approximately 1.5×10^7 leukocytes compared to approximately 1.0×10^8 cells in a B6C3F1 mouse spleen of comparable weight. Pooling of cells, as can be done when syngenic rodents are used, was not an option. Therefore, in order to achieve an adequate yield of splenic lymphocytes for the functional assays, considerably larger fish (200–300 g) were required. Such fish necessitated not only increased animal facility space but often the initiation of new chemical dose-range finding studies. The larger fish appeared to be metabolically different from smaller (45–85 g), but still mature, fish. A caveat is that sensitivity to a chemical may depend on age.

Rather than using the larger fish to test lymphocyte proliferation, the peripheral blood and the pronephros (fish primary hematopoietic compartment, analogous to mammalian bone marrow) were evaluated as alternate lymphocyte sources to the spleen. Peripheral blood collection proved to be unrewarding due to a number of factors. The collection method involved using external landmarks to insert the needle and lacerate the vessels collecting the blood as it pooled within the fish. Problems encountered were: locating the blood vessels consistently, the rapid clotting time of the fish blood and the great variability in blood collection volumes. The most common result of these problems was wide variability in cell quality and recovery. The pronephros was found to be the most reliable source of lymphocytic cells, however, there was considerable contamination of this cell population with other leukocytic cells, including progenitors, which was difficult to overcome. Because commercial monoclonal antibodies were unavailable to phenotype these cells, use of pronephros to evaluate 'lymphocyte proliferation' responses, probably more correctly represented 'leukocyte proliferation' responses. Stress (from handling, temperature variation, and sub-optimal water quality) was another issue which was difficult to measure as well as control. The fish in the model development studies were dosed with chemicals by intraperitoneal injection to allow precise determination of total chemical exposure, but at a cost of increased stress in the fish. Administration of the chemicals in the water would probably have been less stressful but was not an option given that this creates large volumes of contaminated water and thus, a waste disposal problem.

In summary, fish may have some efficacy as models in immunotoxicity risk assessment, however, considerable effort will be required to bring the fish model to the level of the present mouse model. This would include the development of syngeneic lines and production of good commercial reagents and probes (neither of these being trivial endeavors). In addition, it would be preferable to employ one or two species of fish for research purposes to ensure meaningful comparison of data among different laboratories. It will also become essential to increase the present understanding of the physiology and immunopathology of the fish as it compares to mammals. For instance, fish may incorrectly identify rodent (mammalian) immunotoxicants as non-immunotoxic compounds, due to unknown differences in biochemical pathways, etc. Thus, while fish do appear to demonstrate some efficacy for development as a preliminary chemical immunotoxicity risk assessment tool, considerable

effort will be necessary to advance such a new model to the level of the more established rodent models.

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Mini Review

Intracellular oxidation/reduction status in the regulation of transcription factors NF-κB and AP-1

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Received 10 December 1998; received in revised form 16 February 1999; accepted 16 February 1999

Abstract

The eukaryotic cell contains a multitude of pathways coupling environmental stimuli to the specific regulation of gene expression. Two early response transcriptional complexes, NF-κB and AP-1, appear to respond to environmental stressors by inducing the expression of response specific downstream genes. Both are well-characterized transcriptional regulatory factors that are induced by a wide variety of seemingly unrelated exogenous and endogenous agents and serve important roles in cell growth and differentiation, immunity, inflammation, and other preprogrammed cellular genetic processes. The activities of NF-κB and AP-1 are also affected following exposure to chemicals, drugs, or other agents that appear to alter the cellular oxidation/reduction (redox) status. From these observations, it has been suggested that changes in cellular oxidation/reduction status, communicated via a series of cellular redox-sensitive signaling circuitry employing metal- and thiol-containing proteins, serve as common mechanisms linking environmental stressors to adaptive cellular responses. As such, these transcription factors are ideal paradigms to study the mechanism and possible physiological significance of early response genes in the cellular response to changes in cellular redox status. In this article we summarize the evidence suggesting that cellular redox regulates these transcription factors. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Intracellular oxidation/reduction status; Transcription factors; NF-κB; AP-1

1. Introduction

Exposure of eukaryotic cells to adverse conditions is thought to evoke a cellular response that acts in a protective or reparative capacity. Cells

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PII: S0378-4274(99)00024-7

have evolved an adaptive response to environmental stress by initiating a genetically programmed series of events that involves the induction of specific genes (Halliwell and Gutteridge, 1988; Storz et al., 1990). The specific genes activated are dependent on the nature of the environmental stress. For example, heat stress induces a particular set of genes, while UV radiation, ionizing radiation and oxidative stress induce a different set of genes, although there is clearly significant overlap in these responses (Holbrook and Fornace, 1991). A stress-induced response should maintain homeostatic poise, while permitting the cell to exploit and adapt to environmental conditions, thereby minimizing any destructive effects (Storz et al., 1990; Demple and Amabile-Cuevas, 1991). Therefore, cells must sense environmental stress and initiate appropriate signal transduction cascades that result in the activation of specific cellular machinery. Although gene induction is known for some forms of environmental stress, such as DNA damage, comparatively little is known about the mammalian response to genotoxic stress (Rouse et al., 1994).

The response of eukaryotic cells to environmental stress includes the activation of cellular machinery involved in repair, cell-cycle arrest, apoptosis, gene induction, and lethality (Weichselbaum et al., 1994; Maity et al., 1997). In response to certain environmental stressors, such as chemicals, ionizing radiation, phorbol esters, and several chemotherapeutic agents, a class of proto-oncogenes, referred to as early response genes, is activated. These genes were originally characterized in quiescent cells stimulated by the addition of high concentrations of fetal calf serum (Sukhatme et al., 1988; Kerr et al., 1992), and encode transcription factors that bind to specific cis-acting DNA sequences to modulate the expression of other genes (Abate et al., 1991; Karin and Smeal, 1992). Thus, transient induction of these early response genes in response to any number of stimuli, including oxidative stress, may function in coupled short-term changes in cellular phenotype by modulating the expression of specific downstream target genes (Hill and Treisman, 1995). Therefore, knowledge of how the cell regulates these genes in response to stress will contribute to our understanding of cellular oxidative defences and may ultimately lead to the identification of new targets to improve cytotoxic efficacy.

As a result of molecular oxygen in our atmosphere aerobic organisms have evolved using oxygen as an electron acceptor in respiration. Cellular respiration is an extremely efficient method of generating energy in biological systems. However, as side products of electron transfer reactions, aerobic cells continuously produce reactive oxygen intermediates (ROI) from the incomplete reduction of dioxygen molecules (Halliwell and Gutteridge, 1990). ROI, in order of sequential reduction from O_2, include superoxide (O_{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), and organic peroxides, that are normally produced during respiration (Spitz et al., 1992). While low amounts of ROI are natural side products of various electron transfer reactions and easily tolerated by the cell, abnormally high levels of ROI from any number of possible sources induces oxidative stress and can damage cells by peroxidizing lipids, and disrupting proteins and nucleic acids (Spitz and Li, 1990; Sies, 1991; Stevenson et al., 1994). ROI are also produced after exposure to oxidative stress agents, such as UV and ionizing radiation, chemotherapeutic agents, hyperthermia, inhibition of antioxidant enzymes, or depletion of cellular reductants such as NADPH and glutathione (Demple and Amabile-Cuevas, 1991; Holbrook and Fornace, 1991). The cellular response to oxidative stress is of importance since ROI have been implicated as causative agents in several degenerative diseases such as amylotrophic lateral sclerosis, rheumatoid arthritis, and systemic lupus erythematosus, as well as in aging and cancer (Sen and Packer, 1996; Sun and Oberley, 1996; Muller et al., 1997). Therefore, the ability of cells to respond to oxidative stress may be a vital determinant in the subsequent development of these processes.

A wide range of cellular antioxidative molecules and enzymes have evolved to protect and counteract the detrimental effects of ROI produced in the course of cellular respiration (Spitz and Li, 1990). The cellular defense to oxidative stress includes agents or factors that either: (1) detoxify ROI, such as glutathione (GSH), superoxide dismutases

(SODs), and catalase; (2) inhibit cellular reactions that produce free radicals, such as ascorbate and vitamin E; or (3) bind directly to transition metal-containing proteins, such as transferrin or coeruloplasmin (Meister and Anderson, 1983; Spitz et al., 1992). Of these factors GSH, that is mainly present in the cell in the reduced form, appears to provide a crucial pool of reducing equivalents. Reduced GSH in the cell coexists with its oxidized form, GSSG, and the GSH/GSSG ratio is an indicator of cellular stress. Specifically, a decrease in this ratio, either by decreased GSH levels or increased GSSG levels, results in significant oxidative stress. Cellular variations of the GSH/GSSG ratio can have profound effects on the cell in terms of either the activation or inhibition of signal transduction, presumably via induction of specific redox sensitive signal transduction pathways (Ishii et al., 1987; Hwang et al., 1992).

The cellular response to oxidative stress was first observed using *Escherichia coli* exposed to chemicals that produce either H_2O_2 or O_2^-, and this bacterial system provides an excellent model system to explore signal transduction pathways (Storz et al., 1990; Demple and Amabile-Cuevas, 1991). OxyR and SoxR/S, two specific bacterial transcription factors, are induced in response to oxidative stress and activate specific sets of gene families that play a protective role in countering the adverse cellular effects of either H_2O_2 or O_2^- (Halliwell and Gutteridge, 1988; Storz et al., 1990; Demple and Amabile-Cuevas, 1991). OxyR is induced in response to H_2O_2 while SoxR/S is activated following exposure to O_2^- or nitric oxide (NO). The genes controlled by OxyR include catalase/hydroperoxides, glutathione reductase, and alkyl hydroperoxide reductase; the subsequent expression of these genes confers resistance to H_2O_2 (Toledano et al., 1994; Kullik et al., 1995a). Examination of the OxyR protein reveals six cysteine residues, and site-directed mutagenesis has demonstrated one critical cysteine necessary for OxyR activation. Specifically, oxidation of this cysteine residue by H_2O_2 is thought to be the molecular event underlying activation (Storz et al., 1990; Kullik et al., 1995b; Zheng et al., 1998). In this regard, this critical cysteine acts as a redox-sensitive 'sulfhydryl switch' that reversibly modulates activity and defines a novel motif involved in the regulation of DNA-binding activity.

The regulation of SoxR/S and the subsequent expression of the SoxR/S regulon in response to O_2^- appear to involve two distinct stages. SoxR protein is constitutively expressed and is converted by intracellular redox signal(s) to an active form that stimulates transcription of only one known gene, *SoxS*. The SoxS protein binds to and activates all of the SoxR/S regulon promoters (Demple and Amabile-Cuevas, 1991; Wu et al., 1995). The products of the SoxR/S regulon genes mediate a wide variety of cellular resistance to agents that either induce oxidative stress (Greenberg and Demple, 1986; Nunoshiba et al., 1993), such as nitric oxide and superoxide (Nunoshiba et al., 1993, 1995), or against a broad array of antibiotics (Morris et al., 1984). Hence, similar to OxyR, SoxR/S is another example of a bacterial redox-sensitive signal-transduction factor that plays a critical role in the cellular stress response. Since the use of oxygen in cellular respiration is common to all higher organisms, it seems logical that eukaryotic cells would also have genetic pathways to respond to ROI-induced oxidative stress.

As with bacteria the genetic response in mammalian cells to oxidative stress arises when the physiological balance between the production and scavenging of ROI is upset. Excess ROI have been shown to alter the activity of several previously identified early response genes including the transcription factors, NF-κB, AP-1, Erg-l, p53, Ets, and others (Xanthoudakis et al., 1992; Meyer et al., 1993). Much like OxyR and SoxR/S, two transcription factors appear to respond to and protect eukaryotic cells against ROI-induced oxidative stress. These factors, NF-κB and AP-1, are rapidly induced in response to a wide range of agents, factors, and chemicals that alter cellular redox status and both appear to be involved in a variety of pathological conditions (Karin and Hunter, 1995; Kerppola and Curran, 1995). As such, the regulation of these two well-studied inducible transcription factors provides an excellent model system to determine the role of redox status in the regulation of signal transduction.

2. General characteristics of NF-κB regulation

2.1. Components of NF-κB activation

NF-κB is a ubiquitous, pleotropic, multisubunit eukaryotic transcription factor comprised of five subunits referred to as p50, p52, p65 (RelA), c-Rel, and Rel-B that can form homo- and heterodimers in various combinations (Baeuerle and Baltimore, 1996; Verma et al., 1996). NF-κB complexes containing the p65 subunit have been shown to be central in the generation of the immune response. These complexes are activated by antigens, viruses, bacteria, and inflammatory lymphokines, and result in transcriptional initiation of a diverse set of genes whose products are important in the immune and inflammatory responses (Beg et al., 1992; Sha et al., 1995; Besançon et al., 1998). NF-κB is also activated in response to non-inflammatory agents suggesting that NF-κB is involved in cellular processes outside of the immune response (Devary et al., 1993; Henkel et al., 1993). In most cells, NF-κB exists as an inactive heterodimer, the predominant form of which is composed of p50 and p65 (RelA) subunits (Fujita et al., 1993), and is sequestered within the cytosol by association with an inhibitory protein known as I-κB (Karin and Smeal, 1992; Baldwin, 1996). Both the p50 and p65 monomers contain Rel regions approximately 300 amino acids in length, that bind to DNA, interact with each other, and bind the I-κB inhibitors (Zabel and Baeuerle, 1990; Baldwin, 1996). NF-κB activation is post translational and results from the dissociation of the NF-κB:I-κB complex followed by translocation of the released NF-κB into the nucleus (Brockman et al., 1995; Chen et al., 1996). This process leads to increased levels of NF-κB at specific enhancer sites containing the cognate DNA-binding motif 5′-GGGRNNYYCC-3′ (Sen and Baltimore, 1986) resulting in the subsequent activation of target genes, including several inflammatory mediators, cell adhesion molecules, and viral genes (Baeuerle and Baltimore, 1996; Verma et al., 1996).

In the cytosol NF-κB is associated with an inhibitory molecule of the I-κB family and this complex renders NF-κB inactive (Baeuerle and Baltimore, 1988). All six known members of the I-κB family (I-κBα, I-κBβ, I-κBγ, Bc1-3, p100, p105) (Beg et al., 1992; Fujita et al., 1993) contain an ankyrin (ANK) repeat domain that is composed of five to seven closely adjacent repeats required for both association with NF-κB and inhibitory activity (Baeuerle and Baltimore, 1996). Interaction of I-κB with a NF-κB dimer prevents the nuclear uptake of the DNA-binding subunits through the masking of nuclear localization signals (Henkel et al., 1992; Zabel and Baeuerle, 1990). Phosphorylation of I-κB in vivo on serine residues 32 and 36 targets I-κB for protein ubiquitination and subsequent degradation through a proteasome-dependent pathway (Traenckner et al., 1994; Brockman et al., 1995).

While the processes leading to the degradation of I-κB proteins are relatively well understood (Traenckner et al., 1995), the mechanism by which a variety of distinct signals initiated from the cell membrane are transduced to their common targets, the I-κB proteins, remains to be elucidated. A protein kinase activity was recently identified that can phosphorylate I-κB at Ser-32 and Ser-36 (Chen et al., 1996; Lee et al., 1997). This 700-kDa multisubunit complex that was characterized contains kinases that phosphorylate I-κB at serine residues 32 and 36 in I-κB (Chen et al., 1996; Lee et al., 1997). I-κB kinase (IKK) complex activity is induced by TNF treatment, ubiquitin or ubiquitin-conjugating enzymes in vitro, or by mitogen activated protein/extracellular signal-regulated kinase kinase 1 (MEKK1) (Chen et al., 1996; Lee et al., 1997). Fractionation of a similarly sized complex isolated from TNF-treated HeLa cells led to the purification and cloning of the cDNAs of two I-κB kinases, IKKα or IKK-1 and IKKβ or IKK-2 (DiDonato et al., 1996; Zandi et al., 1997). These proteins contain an amino-terminal catalytic domain, a carboxy-terminal helix-loop-helix and leucine zipper domains, that mediate their physical interaction. Exposure of tissue culture cells with TNF increases the kinase activity of both IKKα and IKKβ and identifies IKKα and IKKβ as cellular kinases that inducibly phosphorylate I-κB. Thus, it appears that the I-κB kinase complex consisting of IKKα/β is a critical target molecule for degradation of I-κB and NF-κB activation.

2.2. Redox regulation of NF-κB

NF-κB was one of the first early response genes shown to respond directly to agents that induce oxidative stress (Schreck et al., 1991; Toledano et al., 1994). This was first suggested by the observation that several agents that induce the activity of NF-κB also appeared to induce cellular oxidative stress, as measured by ROI levels or GSH depletion. These agents, that appear to produce ROI and oxidative stress include H_2O_2, TNF, phorbol esters, glutathione depletion, and UV and ionizing radiation (IR), among others (Hayash et al., 1993; Schulze-Osthoff et al., 1995). For example, TNF has been shown to alter mitochondrial electron transport (Schulze-Osthoff et al., 1992); phorbol esters activate NADPH-oxidase in the plasma membrane as well as cyclooxygenase (Haurand and Flohe, 1988; Sun and Oberley, 1996); and ionizing and UV irradiation produce free radicals via emission of valence electrons (Datta et al., 1992; Meyer et al., 1993). These observations indicate that ROI may act as a common second messenger following cellular exposure to any of the multiple agents that induce NF-κB and suggest that specific ROI may be critical and indispensable mediators of the NF-κB signal transduction cascade.

Some of the most compelling work supporting the role of ROI as second messengers in the activation of NF-κB signal transduction pathways was performed by Baeuerle and co-workers using H_2O_2 as a mediator of prooxidant production in culture (Muller et al., 1997). These results clearly demonstrate that treatment of cells with H_2O_2 activates NF-κB DNA-binding, as observed in electromobility shift assays (Schreck et al., 1991; Schulze-Osthoff et al., 1992), and increased gene expression as detected by transient assays using fusion HIV-CAT and 2x-κB TATA-CAT reporter plasmids, confirming that H_2O_2 functionally induced NF-κB transcriptional activity (Schreck et al., 1991). Additional evidence for the role of H_2O_2 as a mediator of signal transduction was obtained using cell lines that overexpress either catalase (which converts H_2O_2 to H_2O) or superoxide dismutase (Cu/Zn-SOD, that converts O_2^- to H_2O_2) (Schmidt et al., 1995a, Schmidt et al., 1995b). In the cell lines that overexpressed catalase, treatment with H_2O_2 failed to increase NF-κB activation, while treatment with H_2O_2 plus aminotriazole, a catalase inhibitor, resulted in increased NF-κB activation. In contrast, cells overexpressing Cu/Zn SOD showed a significant increase or superinduction of NF-κB DNA-binding activity after treatment with H_2O_2. As such, NF-κB appears to respond to the damaging effects of H_2O_2, as well as other strong oxidants and activate a host of eukaryotic transcription factor-sensitive genes that appear to protect the cell against oxidative stress.

These results raise the following intriguing questions: (1) how is oxidative stress sensed by cellular biomolecules and integrated into the genetic pathways that activate NF-κB and (2) where is the common point at which the diverse agents that induce NF-κB and oxidative stress converge? One possibility involves the potential role of the IKKα/β complex as a common and central target molecule. A comprehensive examination of the IKK subunits reveals what appears to be several cysteine residues in the critical kinase domains of IKKα and IKKβ (DiDonato et al., 1996; Zandi et al., 1997). These critical cysteines may act as a redox-sensitive 'sulfhydryl switch' that, via subtle changes in the cysteine redox state, can alter protein conformation and reversibly modulate kinase activity. However, at least two separate protein kinases have been identified that phosphorylate IKKα/β, and it seems likely that other kinases will be identified in the future (Chen et al., 1996; Lee et al., 1997). Thus, how might the IKK complex be a central sulfhydryl switch sensitive to changes in the cellular redox status that can also be regulated by phosphorylation of cytoplasmic kinases? One possibility may involve a mechanism whereby phosphorylation of the IKKα/β results in slight alterations in protein conformation that results in the exposure of a critical cysteine(s). This cysteine(s) thus would be sensitive to very subtle changes in the surrounding microcellular environment or interactions with a cysteine of another redox sensitive protein. Such a mechanism might explain how markedly different agents, via distinct cytoplasmic signal transduction pathways, activate NF-κB and may indicate that the IKK complex serves as a central target switch for activation.

2.3. Antioxidants as inhibitors of NF-κB

In the cell, the balance between the production of free radicals and the antioxidants that scavenge these potentially damaging molecules should, at least in theory, result in a reduced intracellular environment for normal metabolism to proceed. The results above suggest that agents or factors that shift the cellular redox equilibrium to an oxidized state should induce NF-κB, while conditions that sway the balance toward a more reduced state should inhibit the activation of NF-κB. Several laboratories have shown that NF-κB activation is inhibited by various reduced thiol antioxidants, such as NAC (*N*-acetyl-L-cysteine), L-cysteine, 2-mercaptoethanol, aurothioglucose, α-lipoic acid, pyrrolidine dithiocarbamate (PDTC), diethyl dithiocarbamate, and disulfiram; as well as lipid soluble antioxidants and metal chelators such as butylated hydroxyanisol, nordihydroguaretic acid, α-tocopherol, orthophenanthroline and deferrioxamine (Sun and Oberley, 1996; Flohe et al., 1997). These effects are not dependent on any particular structural similarity, suggesting that the antioxidant properties of each molecule may be the common mechanism (Suzuki and Packer, 1993; Makropoulos et al., 1996). Two of these agents, NAC and L-cysteine, increase both the cellular concentrations of GSH and the GSH/GSSG ratio (Kinscherf et al., 1994; Han et al., 1995). This indicates a mechanism whereby reduced thiol pools may alter cellular regulatory pathways in a manner that inhibits oxidation induced cell signals, and provides a common pathway for the role of ROI upon cellular redox status in the regulation of the early gene NF-κB (Fig. 1).

3. General characteristics of AP-1 regulation

3.1. Components of AP-1 activation

The proto-oncogenes c-Fos and c-Jun encode nuclear transcription factors involved in the transmission of inter- and intracellular information

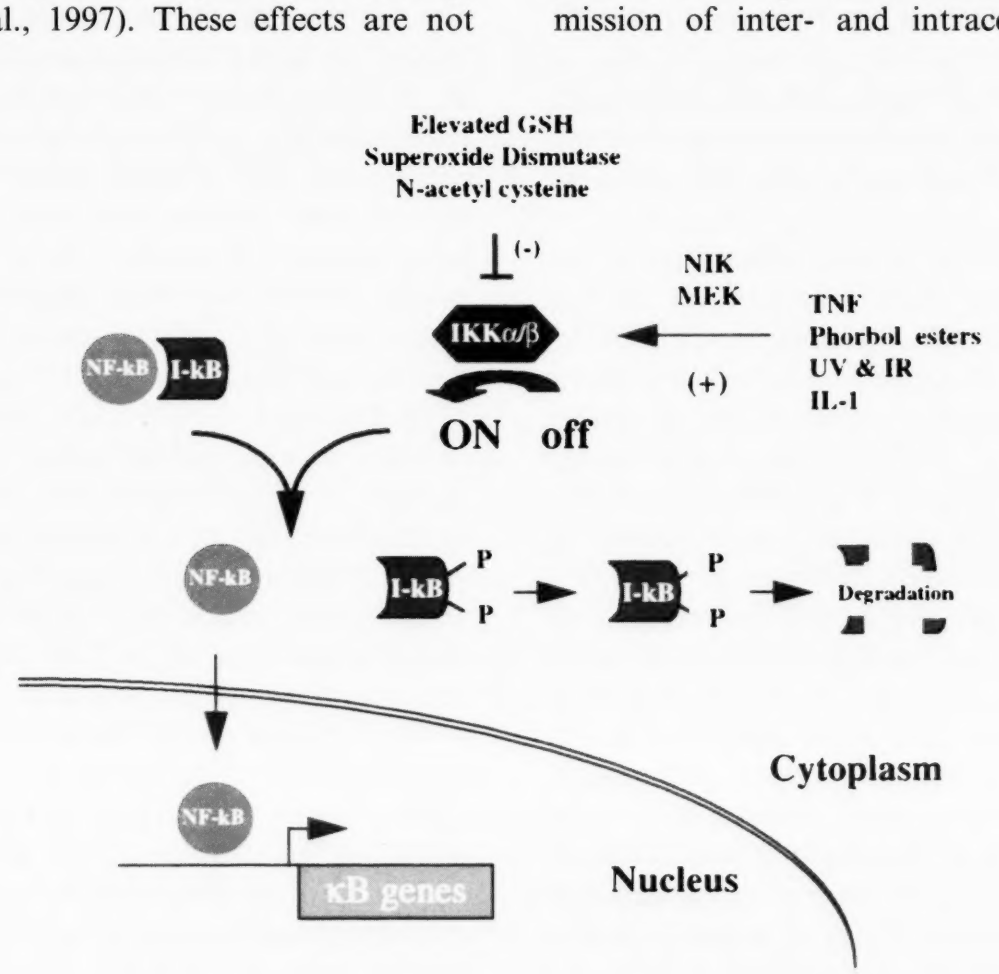

Fig. 1. Regulation of NF-κB composed of p65 and p50.

through multiple signal transduction pathways (Karin and Smeal, 1992; Kerppola and Curran, 1995). c-Fos and c-Jun are members of a multigene family implicated in a number of signal transduction cascades associated with growth, differentiation, neuronal excitation, and cellular stress. As such, they provide a useful paradigm for the investigation of stimulus-evoked alterations in gene expression (Abate et al., 1991; Kerr et al., 1992). AP-1 is a sequence-specific transcription factor composed of either homo- or heterodimers between members within the c-Jun (c-Jun, c-JunB, and c-JunD) and c-Fos (c-Fos, FosB, Fral, and Fra2) families (Angel and Karin, 1991; Kerppola and Curran, 1995). Expression of the various AP-1 factors is differentially regulated temporally during cell-cycle progression and in response to many stimuli (Franza et al., 1988). Given that the various AP-1 proteins have different transcriptional properties as a result of specific activation and repression domains, the composition of the AP-1 complex may be critical to its regulatory function.

AP-1 proteins bind specific DNA sequences, termed TREs (tetradecanoyl phorbol acetate (TPA) response elements), that are present within the regulatory regions of many different genes (Kerppola and Curran, 1995). Binding by c-Jun homodimers or c-Fos/c-Jun heterodimers produces distinct DNA bending resulting in highly specific protein-protein interactions among the AP-1 complex and other promoter-bound transcription complexes (Abate et al., 1991). The c-Jun and c-Fos proteins contain several transcriptionally active regions, including several autonomous transactivation domains, a carboxy-terminal transrepression domain (c-Fos), and a region that interacts with the TATA box-binding protein (Gius et al., 1990; McBride and Nemer, 1998). The exact mechanisms that regulate assembly, targeting, and functional specificity of the different AP-1 complexes remain unclear, although differential gene expression, post translational modification, conformational alterations, and altered DNA-binding specifities of heterodimers may all be involved (Karin and Hunter, 1995; Xanthoudakis and Curran, 1996).

The proteins that make up the AP-1 complex appear to be nuclear rather that cytoplasmic in origin, indicating that other factors must bridge the cellular compartments (Angel and Karin, 1991). As such it appears that cytoplasmic kinase and protein phosphorylation are involved in the activation of AP-1 (Xanthoudakis et al., 1994). Mitogen-activated protein kinase (MAPK) cascades are evolutionarily conserved universal signal transduction molecules involved in a wide variety of biological response mechanisms (Treisman, 1995). The biological effects of MAPK signalling are executed by downstream phosphorylation substrates, most notably a number of signal-responsive transcription factors. The phosphorylation and subsequent induction of c-Fos appears to involve the Raf-1/MAPK pathway and is considered to be a major route for the transmission of signals from Ha-Ras to the c-Fos (Hill and Treisman, 1995).

c-Jun activity can also be directly modulated at the protein level via regulatory phosphorylations occurring on Ser63 and Ser73, and Thr91 and/or Thr93 within the transactivation domain (Pulverer et al., 1991). Phosphorylation of these residues results in the stabilization of c-Jun, as well as enhanced transactivation and DNA-binding activity (Pulverer et al., 1993). c-Jun is also a substrate for a related group of MAPKs, called stress-activated protein kinases (SAPKs), or c-Jun N-terminal kinases (JNK) (Woodgett et al., 1994; Treisman, 1995). Exposure of cells to certain cytokines, protein synthesis inhibitors, or various forms of stress, triggers a kinase cascade leading to the activation of JNK, which can directly bind to and phosphorylate c-Jun (Hai and Curran, 1991). These results demonstrate that phosphorylation plays a central role in the regulation of c-Fos/c-Jun, but in a very different way than with NF-κB (Fig. 2).

In many cell types c-Fos and c-Jun are expressed at low basal levels, but are induced rapidly and transiently by a variety of extracellular signals, including agents or factors that alter the cellular oxidation/reduction status (Abate et al., 1991; Angel and Karin, 1991). As such, c-Fos and c-Jun may function in coupled short-term changes in cellular phenotype by modulating the

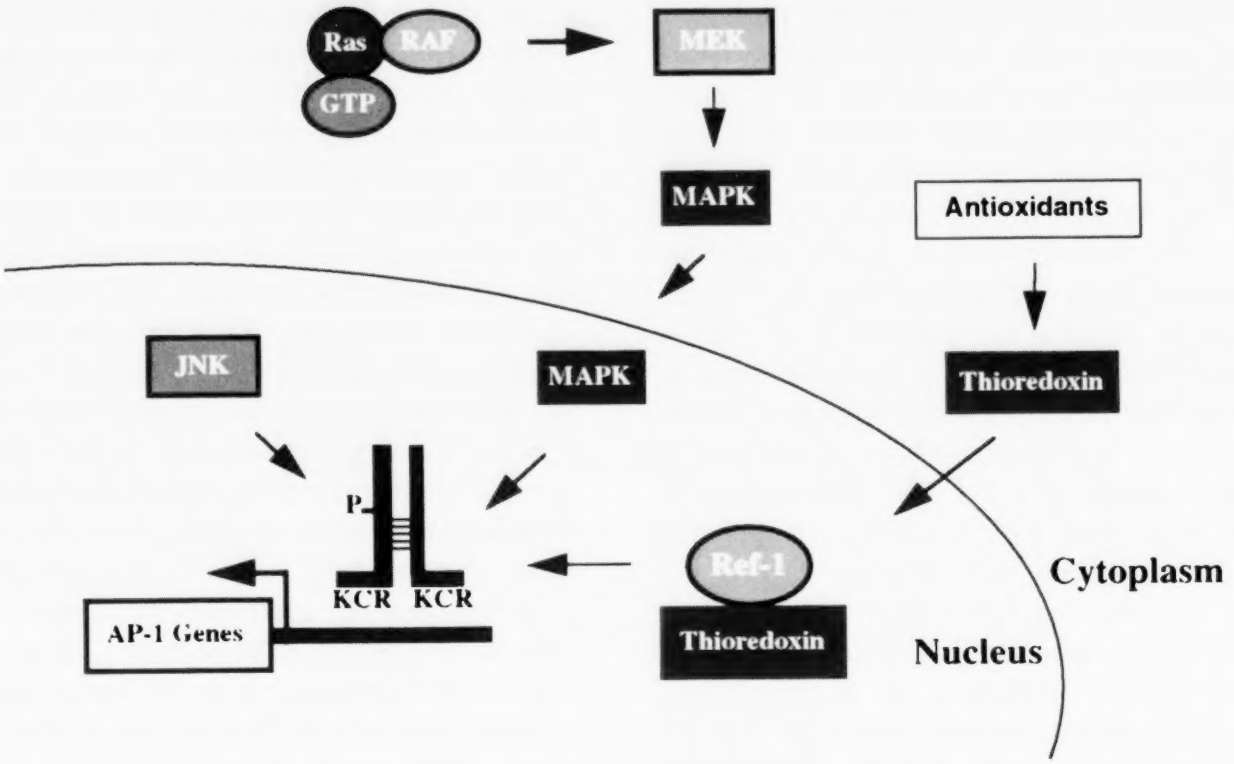

Fig. 2. Regulation of AP-1 composed of c-Fos and c-Jun.

expression of specific target genes (Holbrook and Fornace, 1991; Weichselbaum et al., 1994). A distinct difference between the AP-1 complex and NF-κB lies in the mechanism by which a specific signal is passed from the cytoplasm to the nucleus (Xanthoudakis et al., 1994; Baeuerle and Baltimore, 1996). In the case of NF-κB, the protein complex itself bridges the cytoplasmic nuclear compartments by its transport into the nucleus. In contrast, the c-Fos/c-Jun family proteins are restricted to the nucleus, and alternative pathways involving MAPK appear to pass messages between these subcellular compartments (Karin and Smeal, 1992). In addition to phosphorylation, this may be achieved via a series of redox sensitive factors that will be described below. Thus, redox sensitive proteins may also play a role in how the cell communicates necessary information from one cellular compartment to another in the regulation of cellular processes.

3.2. Redox regulation of AP-1

Similar to NF-κB, it appears that chemicals or agents that alter the cellular oxidation/reduction status also affect the activity of the AP-1 transcriptional complex (Sen and Packer, 1996; Sun and Oberley, 1996). While initial work suggested that oxidants induce AP-1 (Li et al., 1994; Ares et al., 1995; Dalton et al., 1996) it now appears that unlike NF-κB, AP-1 is also strongly induced by some antioxidants (Meyer et al., 1993; Munoz et al., 1996). For instance, in HeLa cells the DNA-binding and transcriptional activities of AP-1 are induced following treatment with PDTC, butylated hydroxyanisole, and NAC (Meyer et al., 1994; Xia et al., 1996) or following the expression of the cytoplasmic antioxidant enzyme system thioredoxin/thioredoxin reductase, all of which increase the GSH/GSSG ratio, resulting in a reduced intracellular state (Schenk et al., 1994; Schulze-Osthoff et al., 1995; Hirota et al., 1997).

Hypoxia is another example of a physiologic process that results in a reduced cellular environment. In this model, there are no O_2 molecules to accept free electrons generated from respiration; hence the flow of electrons in respiratory chains is inhibited, while reducing equivalents increase. However, following reoxygenation there appears to be a rapid increase in prooxidant production resulting in oxidative stress. Work with hypoxia has clearly demonstrated an increase in AP-1

DNA-binding activity during hypoxia, however, following reoxygenation NF-κB is activated presumably due to the rapid formation of oxidants suggesting that NF-κB responds to oxidation while AP-1 to reduction (Yao et al., 1994; Rupic and Baeuerle, 1995).

This raises the interesting question of why under some circumstances the AP-1 complex is induced following oxidative stress and what is the physiological significance of this event. It may be that under certain conditions exposure to an oxidizing agent results in the cell superinducing oxidative stress responsive transcription factors, such as NF-κB, resulting in a compensatory overshoot in cellular redox. Hence the induction of AP-1 may be a response to the cell overcompensating to a more reduced state following oxidative stress, rather than a direct reaction to the damaging effects of oxidative stress.

The levels of c-Fos/c-Jun DNA-binding activity directed towards AP-1 sites in response to multiple environmental stressors can be increased in the absence of de novo protein synthesis via post-translational modification of pre-existing c-Fos/c-Jun protein complexes (Chiu et al., 1987; Angel and Karin, 1991). One post-translation mechanism that modulates c-Fos/c-Jun DNA-binding activity in vitro involves changes in oxidation-reduction state of the protein complex (Abate et al., 1991; Xanthoudakis et al., 1992). In this process, redox regulation is mediated by a conserved cysteine residue, that is flanked by basic amino acids lysine and arginine (KCR), that is located in the basic DNA-binding domain of both c-Fos and c-Jun (Abate et al., 1991). This specific motif (KCR) appears to be conserved in all of the c-Fos- and c-Jun-related proteins and is also present, in a slightly modified form, in several other redox sensitive transcription factors (Xanthoudakis and Curran, 1992; Kerppola and Curran, 1995).

Curran and co-workers have previously shown that changes in the redox state of c-Fos and c-Jun in vitro can alter DNA-binding activity. c-Fos and c-Jun can be converted to an inactive form by chemical oxidation of this cysteine residue and conversely, they can be re-activated by the subsequent chemical reduction of this cysteine (Abate et al., 1991). In vitro DNA-binding activity of c-Fos and c-Jun is enhanced via mutations of these conserved cysteines to serines in the basic DNA-binding domain of c-Fos and c-Jun (c-Fos-C154S and c-Jun-C262S). There is also significant evidence suggesting that redox regulation of c-Fos and c-Jun plays an important physiological role in vivo: (1) naturally occurring cysteine to serine mutations for both c-Fos and c-Jun strongly enhance their transforming ability (Xanthoudakis and Curran, 1996); (2) chemical reduction of c-Fos and c-Jun strongly activate DNA-binding (Abate et al., 1991); and (3) in the absence of chemical reducing agents, a cellular nuclear protein referred to as redox factor-1 (Ref-1) can substitute for reducing conditions and activate the DNA-binding of c-Fos/c-Jun (Xanthoudakis et al., 1992). This suggests that oxidation/reduction is involved in the regulation of DNA-binding, that a cellular protein may transduce this signal to c-Fos/c-Jun, and that the loss of redox transcriptional regulation may be involved with the oncogenetic properties of the Fos and Jun oncogenes.

3.3. The role of redox factor-1 in the regulation of AP-1

Ref-1 (also designated APE, HAP- 1 and APEX), is a class II hydrolytic apurinic/apyrimidinic (A/P) endonuclease that has also been shown to stimulate the DNA-binding activity of AP-1 proteins (e.g. c-Fos and c-Jun) by a redox-dependent mechanism (Xanthoudakis et al., 1992). Ref-1 contains two distinct, functionally independent regions encoding either the redox (N-terminal) or DNA repair (C-terminal) activities. Ref-1 regulates gene expression by reversibly altering the redox state of specific cysteine residues located in the DNA-binding region of several transcription factors, including c-Fos, c-Jun, NF-κB, and Egr-1 (Xanthoudakis and Curran, 1992). Ref-1 was initially purified and cloned based on its AP-1 redox activity, and its DNA repair activity was discovered due to its sequence homology to the DNA repair protein (A/P) endonuclease (Xanthoudakis et al., 1992; Walker et al., 1994). Ref-1 protein purified from HeLa cells stimulates the DNA-binding activity of c-Fos and

c-Jun through the same Cys residues (Fos Cys-154 and Jun Cys-272) shown to be sensitive to chemical oxidation-reduction experiments performed in vitro (Xanthoudakis et al., 1992). Thus suggesting that these critical cysteines, located in the functional domains of redox sensitive proteins, are targets for the passage of redox-sensitive cellular signals.

Since the induction of c-Fos and c-Jun DNA-binding activity following oxidative stress does not require de novo protein synthesis, it would be expected that Ref-1 activity is also regulated via a post-translational mechanism. Recently, it has been demonstrated that Ref-1 activity is also sensitive to changes in the cellular oxidation/reduction. In vitro DNA-binding experiments have shown that bacterially produced Ref-l dialyzed in reduced condition (DTT) strongly activates c-Fos/c-Jun DNA-binding while Ref-1 dialyzed in non-reduced conditions (H_2O_2) inhibited DNA binding (Xanthoudakis et al., 1992). Further genetic analysis of Ref-1 using site directed mutagenesis has identified a cysteine located at position 65 in the redox domain that is critical for the redox-sensitive activation of c-Fos/c-Jun DNA-binding (Walker et al., 1994). Cross-linking studies indicate that this reduced cysteine residue is required for a direct interaction between Ref-1 and c-Jun in vitro (Xanthoudakis and Curran, 1992), suggesting a linear regulatory pathway between Ref-l and c-Fos/c-Jun redox-regulated DNA-binding activity.

Ref-1 activity is also sensitive to reducing factors such as thioredoxin and thioredoxin reductase (Xanthoudakis and Curran, 1996). In the absence of chemical reducing agents such as DTT, both thioredoxin and NAD(P)H activate Ref-1 protein (Xanthoudakis and Curran, 1992; Hirota et al., 1997). Furthermore, in tissue culture cells treated with phorbol 12-myristate 13 acetate (PMA) or ionizing radiation, thioredoxin is imported into the nucleus and forms a physical interaction with Ref-1, implying that Ref-1 participates in relaying a redox signal from the cytoplasm (thioredoxin) to the nucleus to activate nuclear transcription factors (c-Fos/c-Jun) (Hirota et al., 1997; Botero and Gius, unpublished observation.). These findings implicate Ref-1 as a central target molecule in a post-translational redox-sensitive signaling cascade that utilizes thiol-containing proteins to coordinately activate a specific set of redox-sensitive proteins that minimize the cellular damage caused by oxidative stress.

4. Conclusions

Oxidative stress has been suggested to play a role in various clinical diseases, including neurodegenerative disorders, endocrine abnormalities, and cancer. While the understanding of the specific signals that are sensitive to changes in the cellular oxidation/reduction status of the cell is rapidly expanding, the specific mechanism(s) and molecules that bridge the gap between cellular kinases, phosphatases and small redox sensitive molecules remain unclear. However, it appears that many types of factors may use small changes in cellular redox potential/status to induce the activity of early response gene/transcription complexes. One possible mechanism may involve specific cysteine residue(s) located in critical domains of redox sensitive protein kinases, phosphatases, and DNA-binding proteins where the oxidation-reduction potential of a cysteine sulfhydryl ($pK = 8.3$) group is strongly influenced by its local protein microenvironment (Sun and Oberley, 1996). In this model subtle changes in the cellular redox potential alters the ionization state of the cysteine sulfur molecule ($-CH_2-SH$ to $-CH_2-S^-$) resulting in profound changes in protein activity. For example, these regulatory cysteines may not be permissive for activity under oxidized conditions, whereas reduction to a sulfhydryl state would induce protein activity. The critical cysteine(s) would act as a redox-sensitive 'sulfhydryl switch' that reversibly modulates protein activity (Xanthoudakis and Curran, 1996). The idea that redox switching regulates the DNA-binding activity of transcription factors is relatively new (Halliwell and Gutteridge, 1988; Zheng et al., 1998; Abate et al., 1991) and has been received reluctantly due to the general view that the intracellular environment is highly reducing. One explanation supporting this concept is that follow-

ing exposure to ROI oxidized sulfhydryls may exist in discrete microenvironments in the cytoplasm and/or nucleus. This redox potential then could be passed along specific signal transduction cascades using proteins with redox-sensitive sites.

The identification of the oxidant and reductant sensitive molecules remains to be determined but will no doubt provide substantial clarification of the role of cellular redox in the regulation of a host of cellular processes. These processes will likely include the regulation of cellular events outside of signal transduction. For example, it recently has been shown that exposure of tissue culture cells to NAC induces a G_1 arrest (Sekharam et al., 1998). This observation suggests that changes in cellular redox status may be involved in such additional cell processes as cell-cycle checkpoints and, possibly even the regulation of DNA-repair (Sekharam et al., 1998). Finally, increased understanding of the role of ROI and cellular redox status and the determination of the specific signal transduction pathways will further delineate the role of redox in the cellular response to environmental stress and expand the knowledge of a fundamental physiological process, redox regulation of gene expression.

Acknowledgements

The authors would like to thank Drs David Diamond, Jules Harris, Joseph Roti Roti, and Douglas Spitz for providing critical review of this manuscript. We also thank Carla Thurman and Kathy Bles for their assistance with the preparation of this manuscript. D.G. was supported by a grant from the National Institutes of Health (1 K08 CA72602-01).

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Toxicology Letters 106 (1999) 107–118

Toxicology Letters

Mini Review

Mechanisms for xenobiotic transport in biological membranes

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Received 11 January 1999; received in revised form 8 March 1999; accepted 9 March 1999

1. Introduction

Cellular detoxification processes are traditionally classified as phase I and phase II reactions. Phase II reactions are conjugations of the toxicant with one of a number of moieties such as glucuronic acid, glutathione, and sulfate. Usually, conjugation adds to the bulk of the molecule, increases its water solubility, and decreases its reactivity, making the molecule less toxic. For conjugation to occur, an appropriate acceptor group must be present in the toxicant molecule. Examples of suitable functional groups include hydroxyl, carboxyl, and amino groups, epoxides, or reactive double bonds. Many xenobiotics lack such groups and require metabolic activation mediated by phase I enzymes before becoming acceptors for phase II reactions. Benzene is a good example. Being hydrophobic, benzene can partition into membranes and change their properties, but its toxicity would be limited without further metabolism. Other examples of xenobiotics requiring activation include polycyclic aromatic hydrocarbons (PAHs), which are also relatively inert, although they could be mutagenic through intercalation into DNA. For the above compounds, oxidations (prototypical for a phase I process) lead to the addition of polar groups such as hydroxyl groups, or epoxides which render the xenobiotics susceptible to phase II conjugations. However, oxidative phase I processes can lead to the formation of reactive compounds, exemplified by PAH diol epoxides, whose toxicity by far exceeds that of the parent molecule. In these cases, phase II conjugations are obviously of special importance.

Regardless of whether phase I, phase II, or a combination of both reactions is involved, the end products of this process have been traditionally considered to be detoxified and thus not worthy of further attention. This appears to be incorrect

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PII: S0378-4274(99)00061-2

for several reasons. Numerous compounds are activated (Anders and Dekant, 1998), rather than detoxified, even by successive phase I and phase II reactions. In other cases, the product of phase I or phase II detoxification is less reactive, but retains some reactivity, making the detoxification pathway incomplete. An example of the latter situation is the anticancer drug thiotepa, whose glutathione conjugate retains DNA alkylating properties due to the presence of a second functional group (Cnubben et al., 1998). In addition to retaining reactivity, glutathione conjugates can inhibit glutathione *S*-transferases (GSTs), important detoxifying enzymes (Awasthi, et al., 1993). Finally, conjugation with a highly polar moiety such as glutathione traps the reaction product within the cell by preventing its diffusion across biological membranes. Continuing accumulation of conjugates would eventually become detrimental to a cell. Therefore, phase I and/or II detoxification reactions may, somewhat paradoxically, lead to a toxic outcome. Elimination of the products of these reactions from cells is thus an integral part of the detoxification process. It can be achieved by further metabolism, e.g. via the mercapturic acid pathway, or through direct excretion (transport).

The removal from cells of products of phase I or, particularly, phase II reactions through active transport has been termed phase III detoxification (Ishikawa, 1992), and constitutes the topic of the present review. In mammalian cells, the transport occurs across the plasma membrane, while in plant cells metabolic end products are typically concentrated in vacuoles. Interestingly, phase III transport of most, if not all, detoxification products is not only energy-dependent, but is directly coupled to ATP hydrolysis, i.e. is catalyzed by primary transporting ATPases. Possible reasons for such mechanism will be discussed in the concluding remarks of this article. The purpose of this review is to describe mechanisms of detoxification of mammalian tissues through transport mechanisms with a special emphasis on their divergence and the characteristics of multiple proteins involved in these processes. Therefore, in part, this article deals with the still hypothetical matter of origin of these proteins and molecular mechanisms by which these proteins catalyze the active transport of various chemically unrelated compounds such as glutathione conjugates and the anti-tumor antibiotics, such as doxorubicin. In this respect it should be recognized that categorization of these transporters as phase III detoxification is perhaps not correct as some of these transporters can mediate trans-membrane movement of the parent xenobiotics as well as their phase II biotransformation products. It should also be noted that the boundaries between end-stage detoxification and other aspects of metabolism are not sharp. Glutathione conjugation, generally regarded as a detoxification reaction, also serves as a step in biosynthetic pathways, e.g. in the formation of leukotrienes (Scoggan et al., 1997). Likewise, the transporters for excretion of metabolic end products may contribute to both secretion of potentially toxic metabolites and uptake of physiologically important molecules. This applies especially to microorganisms and to specialized secretory organs such as liver. Because it is neither feasible nor, for the sake of a better understanding of the process, desirable to separate the two aspects of transport, both will be discussed in the present review.

2. ABC transporters—structure

The superfamily of ABC (ATP Binding Casette) proteins (Ames et al., 1992; Higgins, 1992; Gottesman and Pastan, 1993; Hrycyna and Gottesman, 1998) is an ubiquitous and evolutionarily ancient group of proteins found in species ranging from bacteria to humans. ABC proteins perform a variety of functions mostly related to transport across biological membranes. The importance of this class of proteins is strikingly illustrated by the fact that almost 5% of the *Escherichia coli* genome is occupied by genes encoding 79 distinct ABC proteins (Blattner et al., 1997; Linton and Higgins, 1998). Similarly, *Bacillus subtilis* encodes 77 putative ABC proteins (Kunst et al., 1997). In both bacterial strains, ABC proteins constitute the largest of the protein superfamilies. Because of their putative defense functions, ABC proteins have been considered to be an ancient cellular 'immune' system (Sarkadi et al., 1996).

In spite of the large diversity in sequence, as well as function and localization, ABC proteins share structural features which are conserved among family members. The minimal structural unit of an ABC protein consists of a transmembrane domain (TMD), usually composed of six transmembrane helices, and a nucleotide binding domain (NBD) (Fig. 1A). The latter includes Walker motifs A and B (Walker et al., 1982).

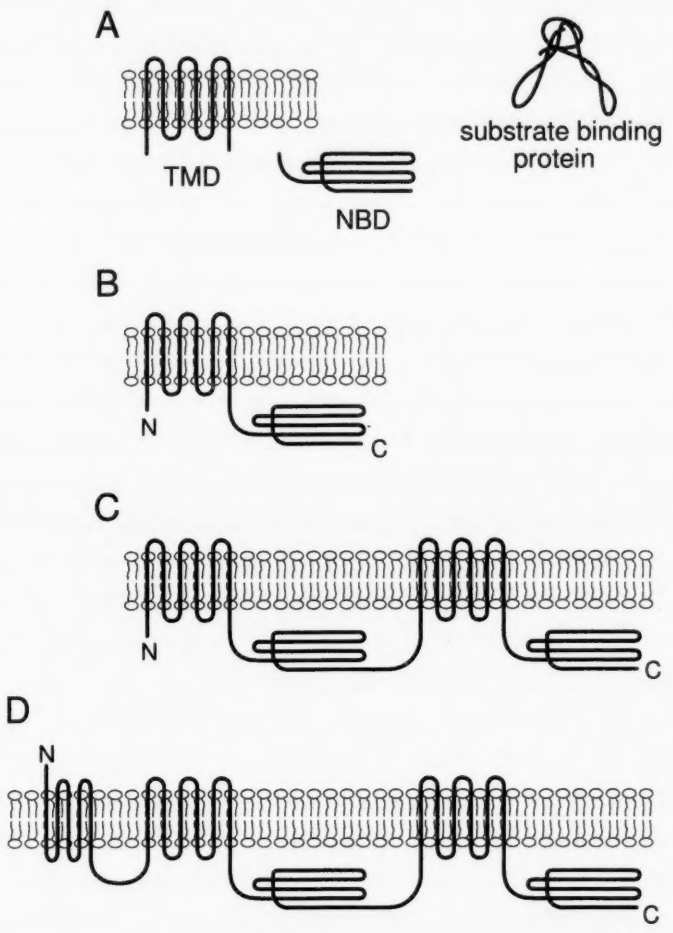

Fig. 1. Schematic representation of structure and membrane topology of ABC proteins. Panel A: bacterial ABC proteins consisting of separate TMDs, NBDs, and, in some cases, additional accessory proteins such as substrate binding proteins and others. These proteins form oligomeric functional complexes of different composition. Panel B: a simple ABC protein in which the TMD and NBD domains are fused; usually, a dimer of such proteins is required for function. Panel C: the structure of a P-glycoprotein (*MDR* gene product) in which further fusion led to a single, functional polypeptide chain. Panel D: the structure of MRP, with the additional N-terminal TMD. In each panel, the area above the membrane corresponds to the topological outside of a cell (which can be the actual extracellular space or the inside of an organelle such as a vacuole), and the area below the membrane corresponds to the cytoplasm.

Walker motif A, or the P-loop, is a glycine-rich loop which binds one of the phosphate groups of ATP, and Walker motif B contains an aspartate residue thought to interact with the nucleotide-associated Mg^{2+} ion (Traut, 1994). These two non-contiguous segments of the protein together form the ATP binding site. While Walker motifs are also found in ATPases and ATP-binding proteins other than the ABC transporters, a characteristic 15-amino acid ABC signature sequence in the NBD domain between the Walker motifs (reviewed by Croop, 1998) has been used for identification and homology cloning of members of the ABC superfamily. In prokaryotic ABC proteins, the TMD and NBD domains occur either as separate proteins, or they may be fused together in various combinations (Nikaido and Hall, 1998). Additional proteins, most notably substrate binding proteins (Fig. 1A), may also participate in the transport event (Nikaido and Hall, 1998). In eukaryotes, the smallest ABC proteins consist of fused TMD and NBD domains, usually in the order shown in Fig. 1B. Strong evidence indicates that such proteins function as dimers rather than monomers (Hrycyna and Gottesman, 1998). In the toxicologically most interesting mammalian ABC transporters, the putative ancestral dimers underwent further fusion to form a single, large polypeptide, as exemplified by P-glycoprotein (Fig. 1C). P-glycoprotein is the product of the *MDR1* (multidrug resistance) gene, and its overexpression is thought to be responsible for cross-resistance of cancer cells selected for resistance to chemotherapeutic drugs. MRP (multidrug resistance-associated protein), another ABC protein associated with multidrug resistance of cancer cells, possesses an additional N-terminal domain (Hipfner et al., 1997; Tusnady et al., 1997) (Fig. 1D), perhaps analogous to a bacterial substrate binding subunit. Thus, ABC proteins seem to exhibit a modular architecture which perhaps is crucial for the flexibility of their functions.

3. ABC transporters—function

Most ABC proteins catalyze ATP-dependent transport of organic compounds, ranging from

small molecular weight molecules to polypeptides. In some instances, ABC proteins regulate the transport function of other proteins as demonstrated by sulfonylurea receptors SUR1 and SUR2A/B, which together with the inward rectifier K^+ channels Kir6.1 and Kir6.2, form a functional K_{ATP} channel (Inagaki et al., 1995). Molecular cloning of SUR revealed that it is a member of the ABC superfamily of proteins (Aguilar-Bryan et al., 1995; Tusnady et al., 1997). It has two NBD domains that contain Walker A and B consensus motifs in which an aspartate residue from the Walker B motif coordinates the magnesium ion of the Mg-ATP, while a lysine residue in Walker A motif is critical for Mg-ADP to be able to activate the K_{ATP} channel activity (Nichols et al., 1996; Gribble et al., 1997).

The most striking feature that differentiates ABC-mediated transport from that catalyzed by other transporters is the curiously wide substrate specificity of ABC proteins. For example, P-glycoproteins transport a variety of drugs and other compounds with no obvious structural relationship to each other, while often excluding compounds with similar structures. This creates a difficulty in conceptualization of the mechanism(s) of ABC-catalyzed transport in terms of conventional enzyme-substrate recognition (reviewed by Sharom, 1997). This conceptual problem is, however, not unique to ABC proteins, and may arguably reflect limitations of our understanding of protein–ligand interactions more than any possible peculiarity of ABC transporters. It has been recently pointed out (Higgins et al., 1997) that structurally well defined binding sites in enzymes may have broad but not promiscuous specificity. GSTs could serve as an example. Human hGSTP1-1 accepts the extremely dissimilar compounds: 1-chloro-2,4-dinitrobenzene (CDNB) and benzopyrene diol epoxide (BPDE) as substrates, while rejecting a closely related stereoisomer of BPDE (Hu et al., 1997a,b; Ji et al., 1997). Nevertheless, the dissimilarity in substrate structures appears greater for ABC proteins than for most enzymes, and additional substrate selection mechanisms have been sought which may act in conjunction with 'classical' binding to an active site.

4. ABC transport mechanisms: P-glycoproteins

Several mechanisms have been proposed to explain the apparent lack of structural similarity between P-glycoprotein substrates. In a radical proposal, it has been hypothesized that drug transport and the resulting drug resistance are secondary to P-glycoprotein-linked changes in membrane potential and pH gradient across the plasma membrane (Roepe, 1995, 1998). This view, however, is not compatible with recent experimental evidence (Sharom, 1998) showing ATP-dependent transport activity by purified and reconstituted P-glycoprotein, which is consistent with the widely accepted idea that P-glycoproteins and ABC proteins in general are ATP-dependent pumps. The structural dissimilarity of P-glycoprotein substrates has directed attention to possible gross physicochemical parameters of the compounds which could determine recognition by the transporter. Substrates of the *MDR1* gene product, the major P-glycoprotein form responsible for multidrug resistance, tend to be hydrophobic or amphiphilic. It has been suggested that only those molecules able to partition into the membrane, or, perhaps more precisely, capable of inserting themselves into the inner (cytoplasmic) leaflet of the membrane, may serve as P-glycoprotein substrates. This would exclude compounds that are highly hydrophobic or hydrophilic (Ueda et al., 1997). In addition, the size of transported substrates must remain between certain lower and upper limits, again excluding vary small and very large molecules (Ueda et al., 1997). These data, together with a low-resolution structure of the P-glycoprotein obtained by electron microscopic image reconstruction (Higgins et al., 1997), support a model (Higgins and Gottesman, 1992; Gottesman and Pastan, 1993; Hrycyna and Gottesman, 1998) in which compounds initially partition into the membrane according to a process determined by their physicochemical characteristics (Fig. 2). From within the membrane, the substrates may bind to P-glycoprotein and either be translocated to the outer leaflet of the membrane ('flippase' activity, Higgins and Gottesman, 1992), or transported across the P-glycoprotein molecule, and directly

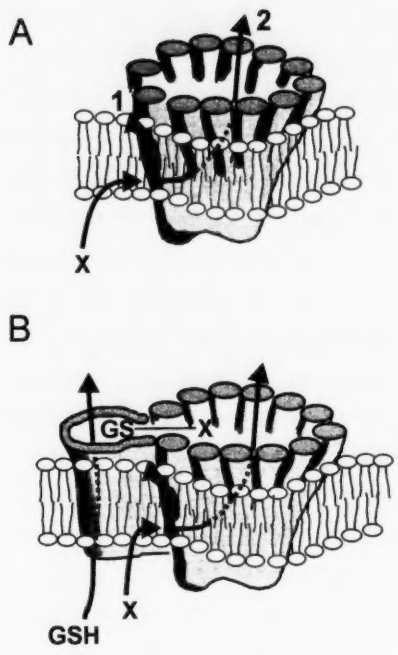

Fig. 2. Cartoon depicting model structures of P-glycoproteins (panel A) and MRP (panel B). The P-glycoprotein structure is based mainly on electron microscope image reconstruction (Rosenberg et al., 1997), and the MRP structure is modeled on the P-glycoprotein structure taking into account the likely membrane topology of MRP (Tusnady et al., 1997). Two possible pathways of substrate transport are shown. In both cases, the substrate first partitions into the membrane. There it is recognized by a binding site on the protein that faces the hydrophobic interior of the membrane. It is thought that conformational changes of the protein brought about by ATP hydrolysis can either cause the substrate to flip to the other leaflet of the membrane from where it could diffuse out of the cell (pathway 1), or it could enter the 'cup' formed by the protein and be ejected into the aqueous phase (pathway 2). In the case of MRP, the fate of a hydrophobic substrate (or the hydrophobic part of a conjugate) may be similar, but the process could be coupled to the transport of glutathione, perhaps through a part of the protein formed by the N-terminal extension shown in Fig. 1D. Alternatively, a covalent conjugate could be translocated in an orientation where the anionic moiety of the conjugate occupies the place of glutathione.

expelled into the external aqueous phase (Higgins et al., 1997). The flippase mode of drug transport by *MDR1* gene product is consistent with the described function of *mdr2* (*MDR3* in humans) gene product, a phosphatidylcholine flippase (Smit et al., 1993). Such a mechanism in which substrates are taken up from the hydrophobic core of the membrane and transported across the P-glycoprotein into the external aqueous phase highlights an interesting aspect of P-glycoprotein function. In conventional channels or ion-transporting ATPases, an important function of the transporter is to shield the inside of the proteinaceous channel from the hydrophobic environment of the membrane (Doyle et al., 1998). In contrast, if this proposed mechanism for ATP-mediated transport is true, the interior of P-glycoproteins would be at least transiently exposed to the membrane. In either case, the substrate binding site of P-glycoproteins appears to be in contact with the core of the membrane rather than open to the intracellular aqueous phase, a postulate supported by the fact that most of the amino acids involved in substrate recognition are located in the transmembrane helices (Ueda et al., 1997).

The idea that substrate selection in P-glycoprotein-catalyzed transport may be determined by gross physicochemical properties of the compounds, primarily hydrophobicity and size, is widely accepted. However, recent data indicate that this may be an oversimplification. Analysis of a large set of known P-glycoprotein substrates has shown that the presence of at least two rather precisely spaced hydrogen bond acceptor groups is required (Seelig, 1998). There is considerable latitude as to the chemical nature of these groups, and the requirement does not appear overly restrictive. Thus, the fundamental principles governing P-glycoprotein-catalyzed transport are beginning to emerge, although much work remains to be done.

5. ABC transport mechanisms: MRP

The mechanisms of transport catalyzed by MRP, another subclass of ABC proteins, is much less understood than that for P-glycoprotein. MRP is of high toxicological interest as it transports not only amphiphilic drugs, but also participates in phase III detoxification as defined by its ability to transport glutathione conjugates, glucuronides, and similar polar compounds. The conceptual difficulty in understanding MRP function is even greater than that in the case of P-glycoproteins. Similar to P-glycoprotein, MRP can accept chemically dissimilar substrates, but these substrates are mostly hydrophilic. Because

of this, attempts to invoke membrane partitioning as a contributing element in substrate selection for MRP (e.g. Sokal et al., 1998) are less convincing than in the case of P-glycoproteins. On the other hand, the broad substrate specificity of MRP which exceeds that of most other enzymes would be most satisfactorily explained by the presence of an auxiliary substrate selection mechanism based on gross physicochemical properties of the substrate rather than on specific structural recognition. The model outlined below uses association of a hydrophobic substrate with a polar moiety as a selection criterion. Since the association may be either covalent or transient, the model could explain the transport by MRP of water-soluble as well as hydrophobic substrates.

A mechanism of MRP function can be postulated by combining recent data on the structure of MRP with results demonstrating a role of glutathione in MRP-catalyzed transport. MRP was originally identified in multidrug-resistant cells which did not express P-glycoproteins (Cole et al., 1992), indicating that, similar to P-glycoprotein, MRP may be able to transport drugs. Later work established that depletion of intracellular glutathione inhibited MRP-mediated transport (Zaman et al., 1995), a finding consistent with the hypothesis that MRP actually transported glutathione conjugates of drugs (Leier et al., 1994; Muller et al., 1994). However, the biological formation of glutathione conjugates of some of the drugs in question (e.g. doxorubicin), has not been reported although such conjugates can be synthesized chemically (Gaudiano et al., 1994; Serafino et al., 1998). Subsequent studies have revealed that MRP is able to transport both conjugated and unconjugated aflatoxin B_1 utilizing similar or overlapping binding sites for the two substrates, and that the transport of the unconjugated form was stimulated by glutathione without the formation of a covalent conjugate (Loe et al., 1997). This indicates that MRP may function as a co-transporter of the particular compound with glutathione; in fact, the co-transport may be obligatory (Rappa et al., 1997). Toxicologists and cancer biologists are primarily interested in drug transport, and thus may view glutathione as merely a co-substrate. However, according to a preliminary report (Paulusma et al., 1997), the specialized hepatic transporter mrp2, originally identified as a canalicular anionic conjugate transporter (Paulusma et al., 1996), may also catalyze the transport of glutathione. This finding is consistent with ATP-dependent glutathione transport by YCF1, a yeast protein belonging to the MRP family (Rebbeor et al., 1998). In liver, the transport of glutathione would be considered the primary physiological function, even if it were coupled to the transport of another compound.

The emerging consensus regarding the function of MRP indicates that the protein may catalyze simultaneous transport of a large, typically hydrophobic molecule such as doxorubicin, and of a polar, anionic moiety, e.g. glutathione or a glucuronate or sulfate group. The two can be covalently linked, as in glutathione *S*-conjugates or glucuronides, can form a complex, as that of glutathione with arsenic compounds or with cisplatin, or could be separate entities. Thus, in contrast to P-glycoprotein, MRP adds the co-transport (probably obligatory) of a negatively charged group or molecule. Since the most striking structural difference between MRP and P-glycoprotein is the presence of an additional N-terminal transmembraneous domain in the former but not the latter (Tusnady et al., 1997) (compare Fig. 1 C and D), it is tempting to speculate that this additional domain is responsible for the translocation of glutathione across the membrane. In this respect, the functional characterization of MOAT-B, a protein highly similar to MRP but lacking the additional N-terminal domain (Lee et al., 1998), will be of considerable interest.

6. Other transporters

The ubiquitous nature and flexibility of function of ABC transporters has attracted the attention of many investigators, and consequently ABC proteins have dominated the field of detoxification related to transport. However, it should be kept in mind that non-ABC systems carrying out similar functions exist, and new ones are likely to be identified in the future. The

transmembraneous movement of phospholipids provides a good example. The identification of the *mdr2* gene product as a hepatic ATP-dependent phosphatidylcholine transporter (Smit et al., 1993) was a major breakthrough in the understanding of biliary physiology, and is often used as an archetype of ABC protein function relevant to normal metabolism. Nevertheless, transport of other phospholipids is catalyzed by an evolutionarily unrelated P-type ATPase (Tang et al., 1996) which, when defective, in humans, results in hereditary benign recurrent intrahepatic cholestasis (Bull et al., 1998). The above findings illustrate a general principle applicable to many enzymes, including detoxification systems. Many such enzymes occur as seemingly redundant, multiple isoforms, usually derived from common ancestral proteins but sometimes arising by convergent evolution. The families of detoxifying enzymes such as cytochromes P450 or GSTs provide abundant examples of homologous proteins. Cytosolic (Armstrong, 1997) and microsomal (Andersson et al., 1994) GSTs illustrate convergent evolution through which unrelated structures assume very similar function. The presence of multiple enzymes with typically overlapping but non-identical substrate specificity may be indicative of a high importance of their function.

6.1. Carcinoembryonic antigen (CEA)

Several reports have linked the behavior usually associated with ABC proteins, (i.e. transport of organic compounds or drug resistance of cells coupled with diminished accumulation of the drug) to a group of proteins exemplified by CEA, a member of the immunoglobulin superfamily. Low accumulation of daunorubicin in leukemic blast cells correlated with the expression of a 95-kDa membrane protein (Doyle et al., 1995) which was later identified as the non-specific crossreacting antigen (NCA) of the CEA antigen (NCA-90), a member of the CEA family of proteins (Ross et al., 1997). Although transfection of cells with NCA-90 has not resulted in multidrug resistance (Ross et al., 1997), expression of CEA itself conferred doxorubicin resistance in a different cell line (Kawaharata et al., 1997). The mechanism by which CEA, or combinations of NCA-90 with CEA, lead to the MDR phenotype is not known, although changes in membrane properties and/or blocking access of drugs to the membrane have been considered (Kawaharata et al., 1997; Ross et al., 1997). However, transport cannot be ruled out, especially in light of a series of communications linking the hepatic cell adhesion protein cCAM105, which has high homology with CEA, to bile acid transport (Sippel et al., 1997 and references therein; Suchy et al., 1997). While this assignment of function has been controversial, and the major hepatic canalicular bile acid transporter has been identified as a specialized form of P-glycoprotein (Gerloff et al., 1998), the repeatedly found link between CEA-like proteins and drug resistance is intriguing and worthy of further study. Additional instances of multidrug resistance possibly connected with lowered drug accumulation, but not due to P-glycoproteins or MRP, have been reported (Lee et al., 1997; Martel et al., 1997a,b), and may reveal new aspects of drug transport.

The cCAM protein implicated in bile acid transport has at least one more known function, namely cell adhesion, and perhaps additional roles, although the connection between cCAM and ecto-ATPase has been questioned (Knowles, 1995). Other membrane proteins with a transport function for a relatively hydrophobic compound in addition to another activity have been reported. Specifically, the microsomal epoxide hydrolase confers sodium-coupled bile acid transport upon cell transfection (von Dippe et al., 1996). Given the parsimony of evolution, the existence of bifunctional proteins is perhaps less puzzling than it initially appears, and is well documented in many systems (Jeffery, 1999). It should not be surprising that this principle may also apply to transport. Conformational changes are an integral part of enzyme function. If the change of shape is sufficiently pronounced to propagate to the surface of the enzyme, and if the enzyme is an integral membrane protein, the conformational change will disturb the protein–lipid interface. This could in turn create a transmembranous pathway for hydrophobic compounds, such as bile acids, which partition into the membrane (see review by

Zimniak and Lester, 1996, for additional discussion of this topic), especially if the protein exhibits some affinity for the substrate. Such interpretation is, in fact, quite similar to the flippase activity envisioned for P-glycoproteins (see above).

6.2. DNP-SG ATPase

A 38-kDa transport protein from human erythrocyte membrane (LaBelle et al., 1988) has been suggested to play a role in phase III detoxification. This protein has an ATPase activity which is stimulated by glutathione conjugates, e.g. 2,4-dinitrophenyl *S*-glutathione (DNP-SG) (hence the designation DNP-SG ATPase) (for review see Awasthi, et al., 1997). Purified DNP-SG ATPase stimulated ATP hydrolysis in the presence of a wide variety of structurally diverse anionic compounds such as DNP-SG (LaBelle et al., 1988), glucuronides, and bilirubin ditaurate (Awasthi, et al., 1991). In addition, the classical substrates of P-glycoprotein (e.g. doxorubicin and colchicine, (Singhal et al., 1991; Awasthi, et al., 1994) also stimulated ATP hydrolysis catalyzed by DNP-SG ATPase, suggesting that this enzyme mediated ATP-dependent transport of these compounds. Purified DNP-SG ATPase reconstituted into proteoliposomes transported doxorubicin as well as DNP-SG in an ATP-dependent, temperature-sensitive manner, against a concentration gradient of these substances across the membrane (Awasthi, et al., 1998a). Similar to ABC transporters, DNP-SG ATPase binds ATP, as indicated by photoaffinity labeling with 8-azido-ATP, and also by binding studies with a fluorescent analog of ATP, trinitrophenyl-ATP (Awasthi, et al., 1998b). Since the transport of doxorubicin and DNP-SG in reconstituted vesicles was mutually inhibitory, a shared mechanism for the transport of these two structurally diverse compounds has been suggested (Awasthi, et al., 1998a,b). The fact that the K_m of DNP-SG ATPase for ATP is in the range of the physiological concentration of ATP, along with its ubiquitous presence in most normal tissues examined to date (Awasthi, et al., 1991; Saxena et al., 1992; Awasthi, et al., 1994, 1998b) suggests that this transporter may be very important in the overall processes of phase III detoxification. This idea is further supported by data showing that its K_m for doxorubicin is in the range of the drug plasma concentrations in patients subjected to doxorubicin therapy (Awasthi, et al., 1994, 1996). Whether DNP-SG ATPase belongs to the ABC transporter family is not known but the available evidence clearly suggests this ATPase to be distinct from P-glycoprotein and MRP.

The remarkably small size of DNP-SG ATPase, in comparison to known human ABC transporters P-glycoprotein and MRP, raises the question as to how the transport of various structurally diverse compounds with opposite charges is mediated by this 38-kDa protein. Among the possible explanations are: (a) the protein itself does not form the whole transport machinery, but rather interacts with other protein(s) to form a functional transporter; (b) the 38-kDa protein may perhaps represent a subunit of a transport complex; or (c) it is a proteolytic fragment of a larger protein, which retains its ATPase and transport activities.

Another transport protein catalyzing organic anion transport has been characterized in liver canalicular membranes (Pikula et al., 1994a,b). The protein was isolated by a combination of ATP, DNP-SG, and lectin-affinity chromatography, and migrated as a single band on SDS-PAGE. The protein had DNP-SG-stimulated ATPase activity and, upon reconstitution into proteoliposomes, catalyzed ATP-dependent transport of DNP-SG against a concentration gradient. Both activities were increased by pretreatment of the protein with protein kinase C. The protein had an apparent molecular weight of 90 kDa (as estimated from SDS-PAGE), and had a small ($\approx 5\%$) polysaccharide content, indicating its distinct nature from mrp2 or cMOAT (Jedlitschky et al., 1994; Muller et al., 1994). Further comparisons of the 90-kDa transporter with ABC proteins will have to await its cloning. However, similarities in kinetic and immunological properties of the 90-kDa protein and the erythrocyte DNP-SG ATPase suggest that the latter two transporters may be related.

7. Concluding remarks

The removal of toxicants from cells, either with or without preceding oxidative or conjugative detoxifying metabolism, appears to be sufficiently important to have driven the evolution of elaborate transport systems. The fact that knock-out animals in which the expression of certain transporters is eliminated fail to show a pronounced phenotype (Borst and Schinkel, 1996) does not cast doubt on the importance of these systems. Instead, such results possibly indicate the presence of multiple transporters with overlapping functions which would not have been maintained in the course of evolution had they not provided the organism with a selective advantage.

ABC proteins appear to be the predominant class of proteins catalyzing transport (along with a number of other processes). However, other proteins carrying out similar functions exist, or their existence is likely. This is not surprising in light of the fact that evolution is opportunistic in the sense of adapting different structures to carry out a required function.

Most, if not all, phase III reactions are ATP-dependent, usually through catalysis by a primary active transporter. This is in contrast to much of cellular uptake of organic compounds, usually nutrients, which is typically coupled to electrochemical gradients of ions such as Na^+ or H^+. Several reasons for this could be envisioned. For hydrophobic or amphiphilic compounds, an assisted flipping across the membrane may be the only mechanistic possibility. If the 'push' is provided by conformational changes of a membrane protein, ATP hydrolysis may be the most straightforward mechanism. Moreover, ion gradients and membrane potential are somewhat unpredictable, especially in unicellular organisms which may find themselves in environments of variable pH or ion concentration. In contrast, a well-regulated level of cellular ATP is one of the universal and probably ancient characteristics of life. The fact that phase III reactions are directly coupled to ATP indicates their early evolutionary formation, and their evolutionary persistence attests to the importance of their function.

Acknowledgements

Supported in part by Grants GM32304 awarded to YCA by the National Institute of General Medical Sciences, and VA Merit Review awarded to PZ.

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Toxicology Letters 106 (1999) 119–127

Toxicology Letters

Glutathione reduces the toxicity associated with antitumor therapy of ascites fluid adsorbed over *Staphylococcus aureus* Cowan I in tumor bearing mice

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Received 23 February 1998; received in revised form 23 November 1998; accepted 25 November 1998

Abstract

It has been well documented in the literature that the removal of circulatory immune complexes (CICs) from the host circulation leads to the immunopotentiation as well as generation of antitumor responses in a variety of tumors in rats, cats, dogs and human patients. CICs are the major immunosuppressive factors in tumor bearing host. Protein A (PA) has been extensively used for the removal of these CICs from the sera/plasma of tumor bearers, because PA has the ability to bind with the Fc portion of mammalian immunoglobulins. Previously, we reported for the first time a potent antitumor response by the inoculation of cell free Ehrlich's ascites fluid adsorbed in vitro over PA containing *Staphylococcus aureus* Cowan I (SAC) in Ehrlich's ascites tumor model. However, there was toxicity associated with this form of therapy in terms of early death of treated animals and the depletion of hepatic glutathione pool as well as phase I biotransformation enzyme and increase in glutathione-*S*-transferase (GST) activities. In the present investigation, tumor bearing animals were treated intraperitoneally (i.p.) on alternate days for 15 days with adsorbed ascites fluid (ad-ASF) (0.1 ml) and glutathione (GSH) (250 mg/kg body weight) separately. We found that GSH supplementation increases mean survival time of GSH and ad-ASF treated mice up to 37.2 days in comparison with 19.9 days for only ad-ASF treated animals, while percent increase in body weight was found to be not affected by the GSH substitution, which remains significantly lower ($P < 0.01$) in comparison to the tumor control animals. GSH supplementation causes a significant decrease ($P < 0.05$) of glutathione-*S*-transferase and restoration of aniline

Abbreviations: AD, aminopyrine-*N*-demethylase; ad-ASF, adsorbed ascites fluid; AH, aniline hydroxylase; EAT, Ehrlich's ascites tumor; GSH, glutathione; GST, glutathione-*S*-transferase; PA, protein A; SAC, *Staphylococcus aureus* Cowan I; TSH, total –SH contents.

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PII: S0378-4274(98)00387-7

hydroxylase activity ($P < 0.05$) and aminopyrine-*N*-demethylase activity. We have also observed that GSH supplementation does not alter the tumor cell viability and tumor cell counts in ad-ASF treated animals in comparison to only ad-ASF treated animals, which indicates that GSH supplementation does not alter the antitumor effect of the therapy. Treatment of Ehrlich's ascites tumor bearing mice with ad-ASF and glutathione increased their survival, but did not reduce the mortality of animals because of tumor. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Glutathione; Antitumor; Adsorption therapy; Protein A; *Staphylococcus aureus* Cowan I

1. Introduction

Tumor bearing hosts are known to be in immunosuppressive state, which is caused by various factors, one of which being the high burden of circulating immune complexes (CICs) in tumor hosts. CICs are the result of incomplete association of antitumor antibodies with tumor antigen. Immune system fails to clear these CICs from the host circulation because of their smaller configuration which results in their accumulation. Accumulation of these CICs causes immunosuppression which supports tumor growth in patients/animals (Ray, 1985). Various reports in the literature indicate that the removal of these CICs from the host circulation causes immunopotentiation and helps in restoring immune competence against tumors in humans as well as in various animal tumor models (Ray et al., 1979; Ray et al., 1980; Ray et al., 1982; Ray, 1985). Removal of CICs also has beneficial effects in various other autoimmune disorders as well as in AIDS (Kiprov et al., 1986; Snyder et al., 1993; Gaddis et al., 1997). Protein A, a 42-kDa cell wall glycoprotein of *Staphylococcus aureus* Cowan I (SAC) is most commonly used for the removal of these CICs, because of its ability to bind more efficiently with the immune complexes in comparison to free IgG only (Forsgren et al., 1983).

Previously, we reported for the first time the antitumor response with inoculation of Ehrlich's ascites tumor bearing mice with cell-free Ehrlich's ascites fluid adsorbed in vitro over protein A-containing *S. aureus* Cowan I. Ascites fluid, in case of ascites tumors is the main source of CICs and a waste, which needs to be removed during the course of treatment (Verma et al., 1999). In that study, a potent antitumor response of the adsorbed ascites fluid (ad-ASF) was observed with concurrent toxicity, a very common feature of antitumor treatments. We observed that the target for this toxicity was the hepatic glutathione pool, resulting in a significant decrease in hepatic glutathione (GSH) contents. There are two possible reasons for this toxicity: (i) many toxic components present in ascites fluid, viz. pseudouridine, 1-methyl adenosine etc., which are of host origin, are produced in increasing amounts with tumor progression (Yamazaki et al., 1973; Tamura et al., 1981; Sami et al., 1986; Takano et al., 1986); (ii) introduction of toxic biomolecules occurs during the process of adsorption over SAC as reported by some investigators (Bandyopadhyay and Ray, 1985).

GSH, a tripeptide γ-Glu-Cys-Gly, is the major source of intracellular thiol groups, and is known to act as a substrate for various toxic metabolites during the course of biotransformation. GSH is also known to reduce the toxicity of various drugs and chemicals and is even used in human cancer patients to reduce the toxicity of anticancer drugs like cisplatin (Anderson et al., 1990; Hamers et al., 1993). In the present study we have attempted to ascertain whether exogenous supplementation of GSH could replenish the depleted GSH levels of the host treated with the ad-ASF therapy without compromising its therapeutic efficacy.

2. Materials and methods

2.1. Animals

Eight-week-old male Swiss albino mice were obtained from the animal breeding colony of the Industrial Toxicology Research Centre, Lucknow,

Table 1
Treatment protocols[a]

Group I	Tumor control	Animals injected with tumor cells and treated with normal saline i.p. on alternate days up to 15 days after the day of tumor inoculation
Group II	Tumor + ad-ASF	Animals injected with tumor cells and treated with adsorbed ascites fluid (ad-ASF) i.p. on alternate days up to 15 days after the day of tumor transplantation
Group III	Tumor + ad-ASF + GSH	Animals injected with tumor cells and treated with both adsorbed ascites fluid (ad-ASF) and GSH (250 mg/kg body weight) separately i.p. on alternate days up to 15 days after the day of tumor transplantation

[a] Animals were injected (i.p.) with 1×10^6 viable tumor cells in 0.5 ml of RPMI 1640. Ad-ASF (0.1 ml) and 0.1 ml of GSH (250 mg/kg body-weight) were injected i.p. separately. There were five animals in each group.

India. Animals were kept in plastic cages (with daily change of sterilized rice husk bedding) and provided with a pellet diet (M/S Hindustan Lever, India) and water ad libitum. Animals were acclimatized for 1 week to the laboratory conditions before the beginning of the experiments.

2.2. Cultivation of S. aureus

S. aureus Cowan I (SAC) was obtained from the American Type Culture Collection (ATCC-12598) and was grown following the methods of Ray et al. (1980).

2.3. Preparation of S. aureus Cowan I suspension

The SAC suspension was prepared by the method described earlier (Ray et al., 1981) with the following modifications. SAC (ATCC-12598) was grown in *S. aureus* Enrichment Broth (Hi-Media, India). The culture was harvested in the log phase (18 h, at 37°C) and the harvested bacteria were washed ($2000 \times g$ for 10 min at 4°C) twice with phosphate buffered saline (PBS, 0.15 M NaCl, 0.1 M phosphate, pH 7.0). The bacterial pellet was suspended in 0.5% formalin in PBS for 3 h at room temperature and washed carefully to remove formalin. Final volume of the bacterial pellet was adjusted to 10% (v/v) with PBS prior to heat attenuation at 80°C for 5 min. The bacteria treated this way have been found to be stable for many weeks.

2.4. Tumors

Transplantable Ehrlich's ascites tumor was procured from the National Institute of Virology, Pune, India and maintained in Swiss albino mice by serial transplantation. The experimental animals were injected intraperitoneally (i.p.) with 1×10^6 viable tumor cells suspended in 0.5 ml RPMI 1640 (Gibco, USA) pH 7.2.

2.5. Collection of Ehrlich's ascites fluid (ASF) and its adsorption

The ascites fluid was collected from the peritoneal cavity of mice which had received an i.p. injection of 1×10^6 viable ascites cells 20 days before. The ascites fluid was centrifuged at $400 \times g$ at 4°C (15 min) to separate the ascites cells from the fluid.

Cell free ascites fluid was mixed with 1:1 ratio of 10% (v/v) heat attenuated, formalin-fixed SAC suspensions and incubated at 37°C for 1 h with slow stirring. This suspension was centrifuged at $2000 \times g$ for 15 min at 4°C to pellet the bacterial mass, and supernatant was stored at −70°C in suitable aliquots after filtering it through a bacterial filter (0.2 μm) under aseptic conditions. The adsorbed ascites fluid (ad-ASF) was inoculated into the host for treatment as mentioned in the treatment protocol (Table 1).

2.6. Viability of tumor cells and total tumor cell counts

On day 18, the ascites fluid was collected from the peritoneal cavity of control and treated animals using an 18G needle. The tumor cells were washed twice with RPMI 1640 and tumor cell counts were done on hemocytometer, and viability was ascertained by using trypan blue dye exclusion test.

2.7. Enzyme assays and –SH contents

The animals were sacrificed on day 18 of ad-ASF treatment, the whole blood and serum were collected and total liver weight was recorded. The liver was homogenized at 4°C with phosphate buffer (0.1 N, pH 7.4) and 10% (w/v) homogenate was prepared using Potter Eivehjem Homogenizer at medium speed setting with ten up and down strokes.

The homogenate was further subjected to sub-cellular fractionation, and 9000 × *g* was obtained using the method of Hook et al. (1972). This fraction was used for the estimation of activity of various mixed-function oxidases. Aniline hydroxylase (AH) and aminopyrine-*N*-demethylase (AD) activities were assayed following the methods of Mazel (1971). Glutathione-*S*-transferase (GST) activity was estimated by the method of Habig et al. (1974) using a molar coefficient of 9.6 for CDNB (1 chloro 2,4-dinitrobenzene). The total –SH (TSH) and GSH contents were estimated by the method of Jollow et al. (1974). Protein contents were estimated by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a reference standard.

2.8. Exogenous administration of glutathione

Reduced glutathione (SRL, India) was freshly prepared in normal saline on the day of administration and 0.1 ml of GSH was given i.p. at 62.5, 125, 250 or 500 mg/kg body-weight with 0.1 ml of ad-ASF separately on alternate days for 15 days from the day of tumor transplantation.

2.9. Treatment protocol

The animals were randomly divided into three groups with 15 animals in each group. Mice in all groups were transplanted with 1×10^6 cells/0.5 ml RPMI 1640 intraperitoneally to raise ascites and their treatment protocol followed as mentioned in Table 1. A total of ten animals in each group were monitored for mortality and body weight changes, and five animals in each group were sacrificed for biochemical studies.

2.10. Statistical analysis

The statistical analysis of data and the level of significance were determined by one-way ANOVA by using Prism software.

3. Results

3.1. Selection of glutathione dose

Initially tumor bearing animals were treated with separate i.p. injections of ad-ASF (0.1 ml) and 0.1-ml different doses of GSH 62.5, 125, 250 and 500 mg/kg body weight in a trial experiment (data not shown). We have observed that 250 mg/kg body weight of GSH treatment gave the optimum protection against the ad-ASF induced toxicity in term of reduced mortality and increase in body weight in comparison to the two lower doses, whereas the next highest dose, i.e. 500-mg/kg body weight dose of GSH, did not show any additional benefit over the 250-mg/kg body weight dose of GSH. Therefore, the 250-mg/kg body weight dose of GSH was selected for further study.

3.2. Tumor growth

A time dependent marked increase was noted in the body weight of all ascites tumor-bearing animals, however the ad-ASF treated animals and animals treated with glutathione in addition to ad-ASF (groups II and III) showed significant ($P < 0.01$) reductions in rate of body weight gain in comparison to group I (tumor control) at 21 days. When the group II and III animals were compared to group I, there were also significant reductions ($P < 0.05$) in percent increase in body weight gained on day 14 (Table 2). A decrease in body weight gain is considered to be an acceptable index for reduction in tumor growth as well as tumoricidal activity of any therapy, and for ascites tumor of peritoneal cavity as reported earlier (Verma et al., 1999).

3.3. Mean survival time

We have calculated the mean survival time

Table 2
Percent increase in body weight of tumor bearing animals treated with ad-ASF and glutathione

Group[a]	Percent increase in body weight[b]		
	Day 7	Day 14	Day 21
I	14.5 ± 1.9	31.9 ± 4.4	73.4 ± 6.3
II	9.7 ± 1.2	20.0 ± 2.9*	28.1 ± 4.6**
III	11.5 ± 1.5	22.3 ± 2.1*	31.7 ± 3.3**

[a] I, tumor control; II, tumor +ad-ASF; III, tumor+ad-ASF+GSH.
[b] Values are mean ± S.E. of ten animals.
* $P<0.05$.
** $P<0.01$.

(MST) for each group of animals on the basis of their survival data. We found that the MST for group III was 37.2 days, significantly higher ($P < 0.01$) in comparison to group I (25.1 days), and group II (19.9 days). GSH supplementation with ad-ASF protects mice from the mortality induced by the treatment (Table 3). This treatment is able to increase the survival of the animals but does not protect from the mortality.

3.4. Change in tumor cell counts and viability

Tumor cell counts were found to be decreased significantly ($P < 0.01$) in groups III and II when compared to group I (Table 4). The decrease in the total tumor cell counts in the peritoneal cavity of the tumor bearing animals treated with ad-ASF is an indication of either slow progression of tumor or relative inhibition of tumor growth.

Table 3
Change in the mean survival time of tumor bearing animals receiving the ad-ASF and glutathione

Group[a]	Mean survival time (days)[b]
I	25.1 ± 0.7
II	19.9 ± 0.7*
III	37.2 ± 0.9*

[a] I, tumor control; II, tumor+ad-ASF; III, tumor+ad-ASF+GSH.
[b] Values are mean ± S.E. of ten animals.
* $P<0.01$.

Table 4
Effect of ad-ASF and GSH on total tumor cell count and its viability in the peritoneal cavity of treated mice

Group[a]	% Viability of tumor cells[b]	Tumor cell count/ml ($\times 10^6$)[b]
I	92 ± 1	204 ± 1
II	68 ± 1*	101 ± 2*
III	62 ± 2*	115 ± 3*

[a] I, tumor control; II, tumor+ad-ASF; III, tumor+ad-ASF+GSH.
[b] Values are mean ± S.E. of five animals.
* $P<0.01$.

Viability of tumor cells as accessed by trypan blue dye exclusion test showed a significant decrease ($P < 0.01$) in groups II and III when compared to group I. The decrease in viability of tumor cells is also indicative of the generation of an antitumor effect of therapy (Table 4). GSH supplementation does not affect the cell viability and the tumor cell counts are suggest that supplementation does not modulate the antitumor effect of ad-ASF.

3.5. Biochemical changes

3.5.1. Changes in biotransformation enzymes

Aniline hydroxylase activity was significantly decreased ($P < 0.01$) in ad-ASF treated mice when compared to the same in tumor control animals. Animals treated with ad-ASF and glutathione (group II) showed a significant increase in the change in the activity of aniline hydroxylase in comparison to group II. Aminopyrine-*N*-demethylase activity was also reduced significantly ($P < 0.01$) in ad-ASF treated mice (group II) in comparison to tumor controls (group I). The enzyme activity showed a recovery in group III when compared to the same in group II (Table 5).

The tumor-bearing animals receiving only ad-ASF (group II) showed significant ($P < 0.05$) elevation in GST levels when compared to the tumor-bearing animals receiving only normal saline (group I), whereas the tumor-bearing animals receiving ad-ASF and GSH showed a significantly lower ($P < 0.01$) decrease in GST activity when compared to group II (Table 5).

Table 5
Percent relative activity of biotransformation enzymes of the tumor bearing animals treated with ad-ASF and glutathione[a]

Group[b]	Aniline hydroxylase	Aminopyrine-*N*-demethylase	Glutathione-*S*-transferase
I	100 ± 7	100 ± 5	100 ± 6
II	62 ± 7**	68 ± 9**	121 ± 4*
III	84 ± 1	83 ± 1	108 ± 4

[a] Control values: aniline hydroxylase, 0.330 ± 0.049; aminopyrine-*N*-demethylase, 2.46 ± 0.44; glutathione-*S*-transferase, 271.28 ± 42.31. Values are expressed as: glutathione-*S*-transferase *n* mol conjugate formed/min per mg protein; aminopyrine-*N*-demethylase *n* mol HCHO formed/min per mg protein; aniline hydroxylase *n* mol *p*-amino phenol formed/min per mg protein; values are mean ± S.E. of five animals.

[b] I, tumor control; II, tumor + ad-ASF; III, tumor + ad-ASF + GSH

* $P < .0.05$.

** $P < 0.01$.

3.5.2. Changes in glutathione contents

The tumor-bearing group of mice treated with ad-ASF (group II) showed significant depletion ($P < 0.01$) of GSH and TSH ($P < 0.05$) contents when compared to tumor-bearing controls (group I). Animals treated simultaneously with glutathione and ad-ASF (group III) had significantly less depletion of GSH contents, in comparison to group II, which suggests the partial uptake of GSH by liver, and may play a role protecting the host from the toxic insult caused by the toxic components present in ad-ASF. We have not observed a significant differences in TSH contents among the different groups as a result of treatment which is an indication for the preferential binding of toxic components of ad-ASF with GSH. This may be a possible reason for the protection of ad-ASF treated mice with the supplementation of GSH, although further studies are required to explain the mechanism of this protection of animals from ad-ASF induced toxic insult. (Table 6).

Table 6
Percent change in hepatic sulfhydryl content of tumor bearing animals treated with ad-ASF and glutathione[a]

Group[b]	Total –SH contents	GSH contents
I	100 ± 7	100 ± 3
II	83 ± 4*	41 ± 8**
III	92 ± 1	73 ± 2

[a] Control values: total –SH contents 33.57 ± 4.16; GSH contents 12.50 ± 2.13. Values are expressed as n mole –SH/g tissue. Values are mean ± S.E. of five animals.

[b] I, tumor control; II, tumor + ad-ASF; III, tumor + ad-ASF + GSH.

* $P < 0.05$.

** $P < 0.01$.

4. Discussion

In an earlier paper we reported a potent antitumor effect of ad-ASF therapy in tumor regressed animals (Verma et al., 1999). At the same time, we observed toxic manifestations including depletion of hepatic GSH contents, enhanced activity of glutathione-*S*-transferase and decreased activity of the phase I biotransformation enzymes like AD and AH levels in ad-ASF treated animals. The toxicity of ad-ASF was also confirmed by the enhanced levels of hepatic and serum glutamate oxaloacetate transaminase and glutamate oxaloacetate pyruvate (unpublished observation), which supports our hypothesis that the toxicity of ad-ASF may probably be related to a hepatic insult caused by the toxic components present in therapeutic material.

In this study, we explored the possibility for the reversal of toxic effect of ad-ASF by the exogenous supplementation of GSH, because it has been established that cytosolic glutathione acts as a nucleophilic substrate during the course of biotransformation of electrophilic toxic metabolites (Lauterburg and Mitchell, 1982). It has been shown that most toxic metabolites produced by different drugs (Mitchell et al., 1974; Brock et al., 1979; Cox and Abel, 1979; Hazelton et al., 1986)

chemicals (Jollow et al., 1974), BHT (Mizutani et al., 1984) as well as aflatoxin (Mgbodile et al., 1975), are electrophilic in nature. These electrophilic metabolites use the cytosolic glutathione pool as a nucleophilic substrate for their detoxification and thereby deplete the glutathione (Gunn et al., 1968). The replenishment of –SH containing amino acid tripeptide, GSH and its precursors had been reported to abrogate the toxicity of various toxicants like BHT (Mizutani et al., 1984), aflatoxin (Mgbodile et al., 1975) and heavy metals (Gunn et al., 1968), and acetaminophen (Mitchell et al., 1974; Benedetti et al., 1975; Wendel et al., 1982). Apart from acting as a substrate for electrophiles, glutathione also protects the host from toxicity caused by de novo generation of hydrogen peroxide (Mills, 1960) and it protects against free radical injury by the formation of thiol radical (Forni and Willson, 1983). Recently, glutathione has been used in clinical trials for reduction in toxicity of various anticancer drugs like cisplatin (DiRe et al., 1990; Hamers et al., 1993).

Glutathione is produced in differential amounts by different cell types, although liver is the major source of its production. The intracellular uptake of GSH from plasma almost always involves its degradation by membrane bound γ-glutamyl transpeptidase, followed by transport into the cell and its resynthesis by γ-glutamylcysteine synthetase and glutathione synthetase. Glutamate is coupled by γ-glutamyl transferase to another amino acid (with a preference for cysteine), and this dipeptide is transported across the cell membrane. If the dipeptide is γ-glutamylcysteine it can be used directly for GSH synthesis by glutathione synthetase (Deneke and Fanburg, 1989). Exogenously administered GSH is reported to increase intracellular GSH levels, but most often this increase can be effectively overcome by the administration of buthionine sulfoxime, a potent inhibitor of γ-glutamylcysteine synthetase. This indicates the importance of the transport mechanism described above. Although this mechanism is one of the most important mechanisms in GSH transport, there is evidence that the tripeptide is taken up directly by a carrier-mediated transport (Kannan et al., 1990). Organs like liver containing high levels of transpeptidase activity are the first to benefit from exogenously administered GSH. The uptake of GSH by hepatocytes is less efficient in comparison to other tissues and organ. Vina et al. (1989) reported that DEM and paracetamol induced hepatic GSH depletion can be replenished by oral GSH in rat and mice. Using this knowledge we tried to reduce the toxicity of this treatment by providing exogenous GSH with the ad-ASF, as we have noticed a decrease in hepatic GSH contents in ad-ASF treated animals (Verma et al., 1999). The supplementation of glutathione, however, did not have any adverse effects on antitumor response as evident from tumor cell counts, viability and body weight gain in ad-ASF and glutathione treated animals in comparison to only ad-ASF treated animals.

The phase I biotransformation enzymes like aminopyrine-*N*-demethylase and aniline hydroxylase showed decreased activity in ad-ASF treated tumor regressor animals. In this study we have selected these two enzymes as an indicator for biotransformation caused by PA as reported earlier in case of CCl_4 and cyclophosphamide. (Dohadwala and Ray, 1985; Srivastava et al., 1987) The exogenous supplementation of GSH along with ad-ASF alleviated the loss of activities of these two enzymes when compared to ad-ASF treatment alone, possibly due to the toxic components of ad-ASF which are detoxified by GSH.

The glutathione-*S*-transferase activity was found to be increased in tumor animals treated with ad-ASF only (Verma et al., 1999). This may be an indication for the production and faster elimination of toxic metabolites from tumor bearing animals treated with ad-ASF. A number of reports indicate the presence of different toxic components like uric acid, pseudo-uridine and 1-methyl adenosine in the ascitic fluid of a tumor bearing host (Yamazaki et al., 1973; Tamura et al., 1981; Sami et al., 1986; Takano et al., 1986). Bandyopadhyay and Ray (1985) have already reported the leaching out of some proteinaceous biomolecules during the extracorporeal adsorption of sera or plasma of human, adsorbed over non-viable and heat attenuated *S. aureus*. Different types of enterotoxins of Staphylococcal origin were also reported to be leached out during the adsorption procedure and were found to be responsible for

toxic symptoms like nausea, chills, fever etc. in human patients (Messerschmidt et al., 1981; Terman and Bertram, 1985). This toxic effect of ad-ASF treatment may be the combined or synergistic effect of one or all of the factors mentioned above.

The antitoxic effect of glutathione did not alter the antitumor effect of ad-ASF as evidenced by a marginal increase in total –SH contents. Simultaneous treatment of glutathione and ad-ASF enhanced the percent survival and mean survival time of tumor-bearing animals without affecting the tumor cell viability and tumor cell counts in group III animals in comparison to group II.

It can be concluded from this study that glutathione may reduce the toxicity associated with the ad-ASF therapy without compromising the antitumor potential of this therapy for Ehrlich's ascites tumor of mice. With further work on the mechanism of the toxicity attenuation by glutathione supplementation, this therapy may become clinically useful for ascites patients. This indicates that there is a wide scope for ad-ASF therapy supplemented with GSH in clinical management of ascites cancer patients at least at the experimental level.

Acknowledgements

The authors thankfully acknowledge the critical review of the manuscript by Professor P.J. Kadowitz, Department of Pharmacology, Tulane University Medical Center, New Orleans, USA. The technical and secretarial help of Ram Vimal and A. Sharma is also thankfully acknowledged. One of the authors (A.S.V.) is appreciative of the award of a junior research fellowship by the Council of Scientific and Industrial Research (CSIR), India.

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Toxicology Letters 106 (1999) 129–136

Toxicology Letters

Effects of hexachlorobenzene on phospholipid and porphyrin metabolism in Harderian glands: a time-course study in two strains of rats

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Received 17 November 1998; received in revised form 19 January 1999; accepted 20 January 1999

Abstract

Hexachlorobenzene, one of the most persistent environmental pollutants, induces uroporphyria and phospholipid alterations in rat liver. Harderian glands produce a secretion that is rich in lipids and accumulate large amounts of protoporphyrin. The aim of the present study was to determine if hexachlorobenzene administration to rats affects phospholipid and porphyrin metabolisms in Harderian glands and if these effects are strain dependent. For this purpose, a time-course study (2, 3 and 4 weeks of hexachlorobenzene treatment) of phospholipid pattern and porphyrin content was performed comparatively in two strains of rats (Wistar and Chbb THOM) which differ in their susceptibility to develop HCB-induced porphyria. Hexachlorobenzene produced decreases in several phospholipid contents, but no changes in phosphatidylcholine levels. While the sphingomyelin/phosphatidylcholine molar ratio remained essentially constant until the third week in Chbb THOM rats, it showed a constant drop in Wistar rats, suggesting a more pronounced alteration of membrane fluidity in the later strain. In regard to porphyrin metabolism, Wistar rats showed an increase in the porphyrin content of the gland, while Chbb THOM animals showed a decrease. The study revealed that not only are the normal parameters of phospholipid and porphyrin metabolism in rat Harderian glands strain dependent, but the response to hexachlorobenzene is also. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Harderian gland; Hexachlorobenzene; Phospholipid; Porphyrin; Rat

Abbreviations: ALA, δ-aminolaevulinic acid; HCB, hexachlorobenzene; PBG, porphobilinogen; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPM, sphingomyelin.

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PII: S0378-4274(99)00015-6

1. Introduction

Hexachlorobenzene (HCB) is a highly lipophilic chlorinated hydrocarbon, formerly used as a fungicide. Nowadays, it is produced as a by-product or impurity in the manufacture of chlorinated compounds and of several registered pesticides. It is among the most persistent environmental pollutants, and bioaccumulates in the environment, in animals and humans (Courtney, 1979; Wilson and Kueberuwa, 1994).

In rats, HCB induces an experimental uroporphyria that resembles human cutanea tarda porphyria, with the liver as its major site of action (for review see De Matteis, 1991). This effect has been seen to be dependent on the strain of rat. The Chbb THOM strain shows much lower susceptibility to acquire HCB-induced porphyria than animals of the Wistar strain (Wainstok de Calmanovici et al., 1989).

The heme metabolic pathway is not the only pathway to be affected by HCB. It has been reported that HCB alters the fluidity of liver membranes by altering their phospholipid composition (Kószó et al., 1982; Cantoni et al., 1987). In this regard, we have reported that Wistar rats treated with HCB over a 1–8-week period showed alterations in hepatic phospholipid metabolism. All phospholipids, with the exception of sphingomyelin, presented a biphasic behaviour. The turning point between both phases was the time at which porphyrin levels increased, suggesting a relationship between both metabolisms (Billi de Catabbi et al., 1997).

Harderian glands are large orbital structures, that are especially prominent in rodents. They produce a secretion that is rich in lipids, and accumulate large amounts of protoporphyrin due to a low level of ferrochelatase and high levels of the other enzymes involved in heme biosynthesis (Spike et al., 1992; Nagai et al., 1997). These characteristics make them an attractive organ to study the control of the synthesis of both porphyrins and phospholipids.

In the female golden hamster, the apical parts of tubule epithelial cells possess numerous lipid vacuoles, and the luminal contents of the gland consist of lipid vacuoles (often with intact membranes) and large solid accretions of porphyrin. It has been suggested that these porphyrin accretions are probably complexed with lipids (McGadey et al., 1992).

A number of studies have analyzed the lipid content of rodent Harderian glands, and found species and sex differences (Bareggi et al., 1979; Lin and Nadakavukaren, 1981; Seyama et al., 1992). However, to the best of our knowledge, there has been no quantitative study of the Harderian gland phospholipid composition in different strains of rats, nor of the effects of porphyrinogenic drugs on the lipid composition of these glands.

It has been reported that following HCB administration, the rat Harderian gland contains a high concentration of the fungicide, and that it forms part of the excretory pathway of HCB (Ingebrigtsen and Nafstad, 1983). Studies in our laboratory showed that long-term HCB treatment of Wistar rats, alters the heme metabolism in the Harderian gland, but that the alterations observed differ from those found in the liver (San Martín de Viale et al., 1975, 1977). In this sense, it has recently been proposed that the regulation of heme biosynthesis in Harderian gland might be different from that in the liver (Nagai et al., 1997).

The aim of the present study was to determine if short-term HCB administration to rats affects phospholipid and porphyrin metabolisms in Harderian glands and if these effects are strain dependent. For this purpose, a time course assay (2, 3 and 4 weeks of HCB treatment) of phospholipid composition and porphyrin content was performed comparatively in two strains of rats (Wistar and Chbb THOM) which differ in their susceptibility to develop HCB-induced porphyria.

2. Materials and methods

2.1. Materials

HCB (commercial grade) was generously provided by Compañía Química (Argentina). High quality supplies of: phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), and sphingomyelin (SPM), as well as precoated thin-layer chromatography (TLC) plates (particle size 2–25

μm, thickness layer 200 μm, on an aluminum support), were purchased from Sigma (USA). All other chemicals were of the highest available grade.

2.2. Animals and treatments

Female Wistar and Chbb THOM rats of the same age, weighing 160–180 g at the start of the experiment, were used. Procedures involving animals (care and use) were conducted in conformity with international guidelines (Guide for the Care and Use of Laboratory Animals, National Research Council, USA, 1996, and the Council of the European Communities Directive, 86/609/ECC).

The rats were maintained on a 12-h light/dark cycle and fed Purina 3 diet and water, ad libitum. Animals of each strain were randomly assigned to two groups: (1) controls ($n = 15$), and (2) those treated with HCB ($n = 15$). HCB was administered 5 days/week by stomach tube. Each day the animals received a single dose of HCB (1 g/kg per day) suspended in water (40 mg/ml) containing Tween 20 (0.5%). Animals from the control group received the vehicle alone. An additional group of six animals of each strain received no treatment and was used to establish the normal phospholipid composition of Harderian glands (Table 1).

The dose of HCB used in this work was chosen based on previous studies demonstrating that this dose elicits clear manifestations of hepatic porphyria in Wistar rats, with no deaths or external alterations (San Martín de Viale et al., 1970, 1975, 1977; Wainstok de Calmanovici et al., 1989; Billi de Catabbi et al., 1997; Randi et al., 1998). The low order of toxicity is indicative of the minimal absorption of HCB across the intestinal tract when administered in water. In this sense, it has been shown that [^{14}C]HCB suspended in water and administered intragastrically is very poorly absorbed, estimated at 5% of the dose (Koss and Koransky, 1975; Courtney, 1979).

A long delay time in the manifestation of porphyria is typical of HCB-induced porphyria. We have observed that Wistar rats show a statistically significant increase in the hepatic porphyrin content only after 3 weeks of HCB treatment (Billi de Catabbi et al., 1997). A time-range around the third week of treatment was chosen for this study and so, the animals were sacrificed by decapitation between 08:00 and 09:00 h following 2, 3 and 4 weeks of treatment.

2.3. Preparation of tissue sample

Harderian glands of the rat are highly irregular and cover much of the posterior surface of the eyeball. After removal of the eyeball, Harderian glands from each animal were rapidly excised, rinsed with cold 0.9% NaCl, blotted on filter paper and weighed. The average weight of both glands was 0.257 ± 0.009 and 0.245 ± 0.010 g for Wistar and Chbb THOM control rats, respectively.

Table 1
Normal phospholipid composition of Harderian glands from Chbb THOM and Wistar rats[a]

Phospholipid	Chbb THOM		Wistar	
	Phospholipid content (nmol Pi/mg tissue)	%	Phospholipid content (nmol Pi/mg tissue)	%
PE	9.70 ± 0.36	23.3 ± 0.9	7.20 ± 0.60*	21.8 ± 1.8
PS	1.25 ± 0.14	3.0 ± 0.4	1.30 ± 0.24	3.9 ± 0.7
PI	3.45 ± 0.28	8.3 ± 0.7	2.75 ± 0.15*	8.3 ± 0.5
PC	24.00 ± 0.98	57.8 ± 2.3	18.75 ± 1.10*	56.7 ± 3.0
SPM	3.17 ± 0.14	7.6 ± 0.3	3.06 ± 0.22	9.3 ± 0.7
Total	41.57 ± 1.10	100.0 ± 2.6	33.06 ± 1.30*	100.0 ± 5.0

[a] Lipids were extracted and separated by thin layer chromatography and phospholipids were individually quantitated as described in Section 2. PE, phosphatidylethanolamine; PS, phosphatidylserine PI, phosphatidylinositol; PC, phosphatidylcholine; SPM, sphingomyelin.Data are mean values ± S.E.M. of six animals.

* Statistically different from Chbb THOM rats ($P < 0.05$).

2.4. Porphyrin content

Porphyrin content was determined spectrophotometrically in 50-μl portions of 10% (w/v) homogenates in 0.154 M KCl as described by San Martín de Viale et al. (1977).

2.5. Phospholipid determination

Total lipids were extracted in 100 μl of 10% (w/v) homogenates in 0.154 M KCl, according to Bligh and Dyer (1959). Phospholipid separation was performed on precoated thin-layer plates by one-dimensional, two-solvent system, thin-layer chromatography, as described by Sterin-Spezziale et al. (1992). The first solvent system used was a mixture of chloroform–methanol–acetic acid–water (40:10:10:1, v/v). Plates were dried and developed to a level 0.6 cm below the first solvent front in a solvent system consisting of chloroform–methanol–acetic acid–water (120:46:19:3, v/v). Lipid fractions were detected with I_2 vapor. Specific areas of the plates were scraped off and digested with 70% perchloric acid in the presence of ammonium molybdate (0.5%), and the resulting inorganic phosphate was assayed with the Fiske Subarow reagent (Bartlett, 1959).

2.6. Statistical analysis

Results are expressed as means ± S.E.M. Student's *t*-test was used to assess differences in normal phospholipid composition between Wistar and Chbb THOM rats. Data on porphyrin content and phospholipid composition in the time-course studies were submitted to a one-way ANOVA. In each analysis, the *F*-test was used to determine statistical significance. In all cases 0.05 was used as the level of significance.

3. Results

3.1. Porphyrin content

Assays of porphyrin content in the Harderian glands of Wistar and Chbb THOM rats showed interstrain differences (Fig. 1). Control Wistar rats exhibited a lower porphyrin content than Chbb THOM animals, though the difference was not statistically significant. In Wistar rats, HCB treatment promoted significant increases in porphyrin content by the second and third weeks of treatment (46 and 58%, respectively), returning to control values by the fourth week. In contrast, Harderian glands from Chbb THOM rats showed important decreases in porphyrin content throughout the experiment (57, 67 and 55%, for the second, third, and fourth weeks, respectively).

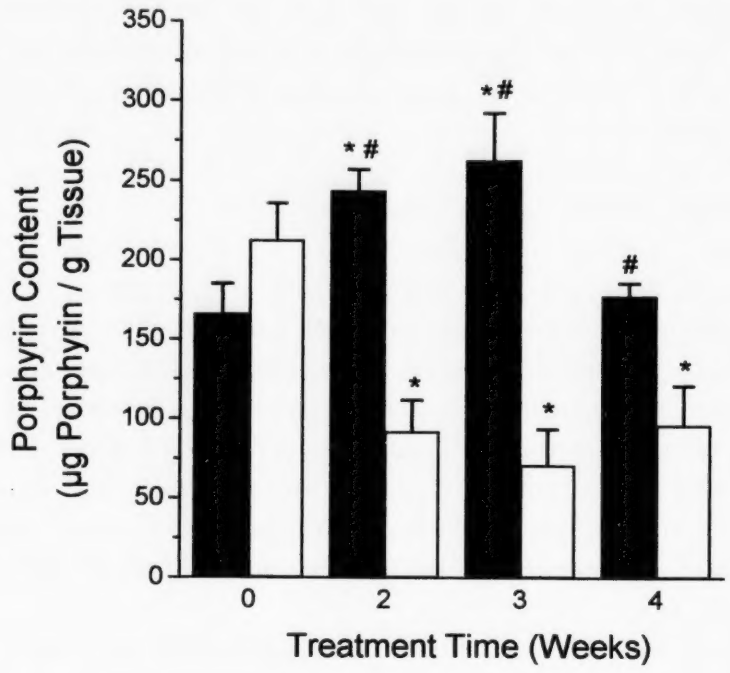

Fig. 1. Time-course effects of HCB on porphyrin content in Harderian glands of Wistar and Chbb THOM rats. Control values were not altered by vehicle administration up to 4 weeks. Each bar represents the mean ± S.E.M. of five animals. Wistar (black bars), Chbb THOM (open bars). *Significantly different from control rats; # significantly different from time-matched HCB-treated Chbb THOM rats ($P < 0.05$).

3.2. Phospholipid analysis

3.2.1. Normal phospholipid composition

Table 1 presents the phospholipid distributions from normal Harderian glands of Wistar and Chbb THOM rats. In both strains, PC was found to be the main phospholipid, followed by PE, while the lowest levels corresponded to PS.

Interstrain differences in individual phospholipid contents were observed. PC, PE, and PI levels, measured as nmol Pi/mg tissue, were lower

in Wistar than in Chbb THOM rats. This accounts for the 20% lower total phospholipid content observed in Wistar rats.

The SPM/PC ratio is an accepted determinant of membrane fluidity. In this respect, Chbb THOM rats showed a SPM/PC molar ratio lower than Wistar animals (0.132 and 0.163, respectively), suggesting a constitutive difference in membrane microviscosity between the two strains.

It is worth mentioning that the PS/PE molar ratio was also different for both strains (0.129 and 0.180 for Chbb THOM and Wistar Harderian glands, respectively).

3.2.2. *Effects of HCB on individual phospholipids*

3.2.2.1. Wistar rats. The effects of HCB on individual phospholipids in the Harderian glands of Wistar rats are shown in Fig. 2. It can be seen that HCB treatment produced changes in all the phospholipids studied with the exception of PC, which remained essentially unchanged during the course of the experiment. SPM levels were significantly decreased throughout the study, resulting in a drop in the SPM/PC molar ratio (0.139, 0.134, and 0.139 at weeks 2, 3, and 4, respectively, versus 0.163 for controls).

With regard to the other phospholipids, an early significant decrease in PE and a slight increase in PS were observed in the second week of treatment. In the third week, PE was restored to normal values, while PS began to decrease. These changes in PE and PS levels resulted in variations in the PS/PE molar ratios (0.239, 0.147, and 0.167, at weeks 2, 3, and 4, respectively, versus 0.180 for controls).

Significant changes in PI levels were seen only by the third week of treatment with the fungicide.

3.2.2.2. Chbb THOM rats. Fig. 3 presents the effects of HCB on the different phospholipids in Harderian glands of Chbb THOM rats. Similarly to what was observed for Wistar, PC levels in Chbb THOM rats were unaffected throughout the treatment. SPM levels were significantly diminished by the end of the experiment and, consequently, the SPM/PC ratio remained essentially constant until the third week, showing a decrease only by the fourth week (0.110 at week 4 versus 0.132 for controls).

An early decrease in PE was observed, but, in contrast to Wistar rats, the levels of this phospholipid were not restored until the fourth week. PS levels were drastically decreased by about 42% below control values, by the third week of treatment, and an important loss in the contents of this phospholipid was also observed in the fourth week. This altered pattern of PS and PE levels produced variations in the PS/PE molar ratios (0.164, 0.087, and 0.085, at weeks 2, 3, and 4, versus 0.129 for controls). It is worth pointing out that the variations in the PS/PE molar ratio were more important in Chbb THOM than in Wistar rats.

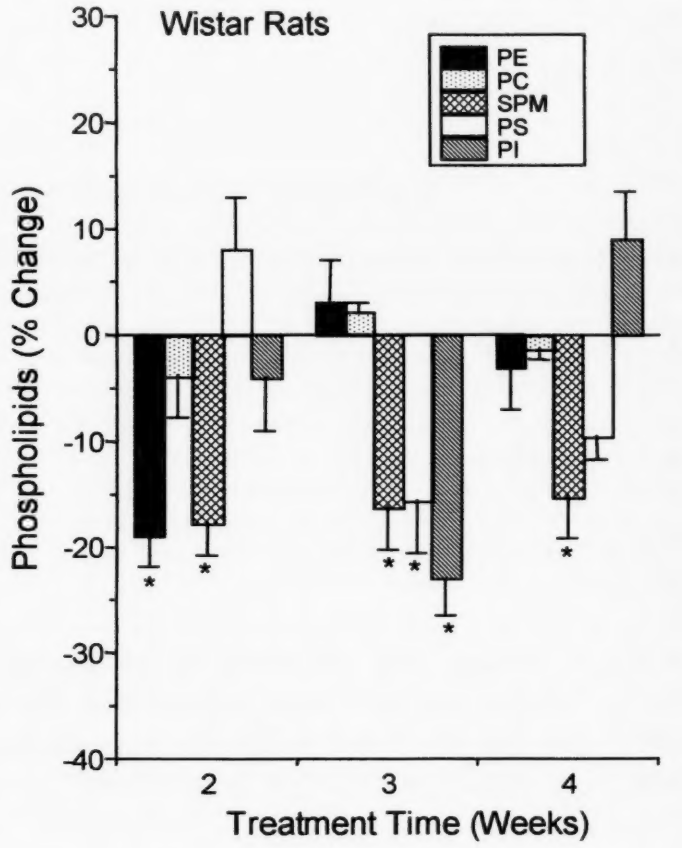

Fig. 2. Time-course effects of HCB on individual phospholipids in Harderian glands of Wistar rats. Results show the changes in percentage of each phospholipid type compared to the corresponding control values. Lipids were extracted and separated by thin-layer chromatography and phospholipids were individually quantitated as described in Section 2. PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SPM, sphingomyelin. Data are mean values ± S.E.M. of five animals. *Significantly different from the corresponding control values ($P < 0.05$).

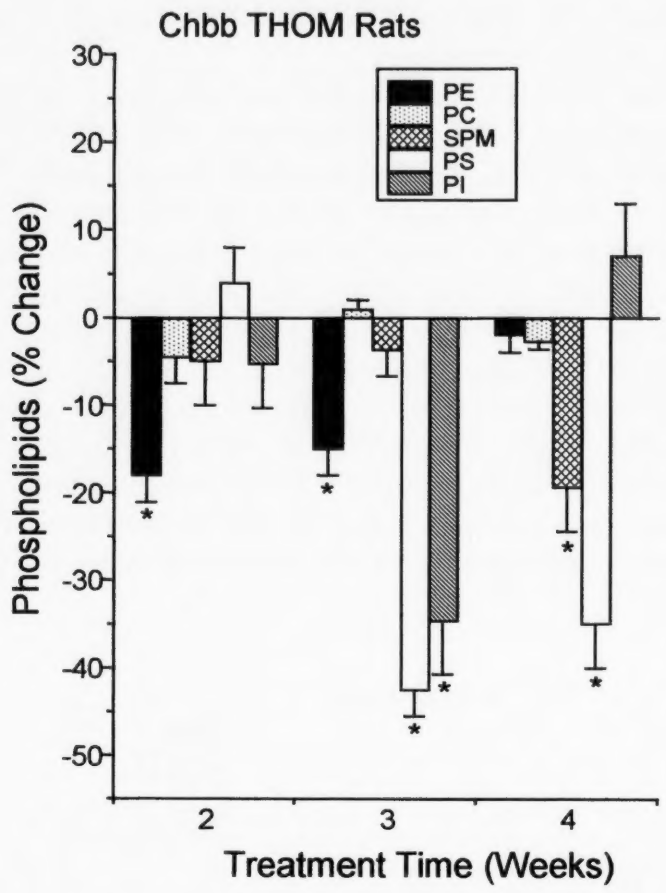

Fig. 3. Time-course effects of HCB on individual phospholipids in Harderian glands of Chbb THOM rats. Results show the changes in percentage of each phospholipid type compared to the corresponding control values. Lipids were extracted and separated by thin-layer chromatography and phospholipids were individually quantitated as described in Section 2. PE, phosphatidylethanolamine; PS, phosphatidylserine PI, phosphatidylinositol; PC, phosphatidylcholine; SPM, sphingomyelin. Data are mean values ± S.E.M. of five animals. *Significantly different from the corresponding control values ($P < 0.05$).

As in Wistar rats, PI levels in HCB-treated Chbb THOM animals were significantly diminished only by the third week, but this decrease was more pronounced in the latter strain.

4. Discussion

In the present study, we found that phospholipid metabolism in the Harderian gland of HCB-treated rats was altered in the two strains of rats studied. There was a decrease in the contents of several phospholipids and no significant increases during the intoxication period studied. The absence of an increase in phospholipid content could indicate a lack of endoplasmic reticulum proliferation, as a consequence of HCB metabolism. In this regard, the enhanced phospholipid content produced by HCB and other cytochrome inductors in the liver, has been reported to be an indicator of proliferation of endoplasmic reticulum membranes (Ishidate and Nakazawa, 1976; Billi de Catabbi et al., 1997).

HCB affected the molar ratio between PS and PE contents in both rat strains, though the effect was greater in the Chbb THOM strain. Taking into account that, in mammalian cells, the biosynthesis of PE is linked to a cyclic reaction between PE and PS, catalyzed by base exchange enzymes and phosphatidylserine decarboxylase, it can be postulated that HCB may be deregulating this cyclic reaction. However, an increase in phospholipase activities in order to repair HCB damage of membrane phospholipids, should not be disregarded.

It has been previously described that HCB treatment of Wistar rats causes a time-course decrease in liver SPM contents (Billi de Catabbi et al., 1997). The present study shows a similar response in the Harderian gland of only Wistar rats. In contrast, animals of the Chbb THOM strain showed more resistance to the impairment of SPM metabolism, suggesting that the regulation of choline-containing phospholipids, and thus the regulation of membrane permeability, is more efficient in this strain than in Wistar rats.

With regard to heme metabolism, the data presented here show, for the first time, strain differences in normal porphyrin concentration in rat Harderian gland. Such strain differences have been reported previously for mice (Shirama and Hokano, 1992; Spike et al., 1992). In addition, we found that HCB effects on heme metabolism in Harderian glands are also strain dependent, since Wistar and Chbb THOM rats showed opposite responses: in Wistar rats, HCB elicited an increase in the porphyrin content of the gland, while in Chbb THOM it caused a significant decrease. These differences in porphyrin content could be related to interstrain differential behavior in response to HCB treatment of δ-aminolaevulinic acid dehydrase and porphobilinogen deaminase.

These enzymes are involved in porphyrin biosynthesis and are known to be affected by HCB in this tissue (San Martín de Viale et al., 1975).

Changes in total porphyrin content in the Harderian gland may be the result not only of changes in the biosynthesis pathway, but also of changes in the excretion of stored intra-luminal porphyrin or of interstitial porphyrin. In this respect, it is known that the Harderian gland contributes porphyrins to the circulation and it has been reported that tubule wall degeneration within the gland leads to relocalization of porphyrins within the interstitium, accompanied by elevated blood porphyrin levels (Payne, 1994). It has also been observed that the excess of gland porphyrins produced in rats by the porphyrinogenic compound allylisopropylacetamide was excreted onto nose and whiskers (Wetterberg et al., 1971). In addition, it seems to be a link between phospholipid biosynthesis and porphyrin deposits within the gland. Thus, it has recently been observed that the chronic administration of CDP-choline, a precursor of the membrane lipid PC, alters the gland composition by augmenting the number of lipid vesicles and preventing porphyrin deposition (Verduga et al., 1997). Consequently, the strain differences in porphyrin content observed in this study following HCB treatment, may be related to the changes in membrane permeability that we found were strain dependent. Nevertheless, further studies are needed to clarify this point.

In summary, we have found strain-dependent differences in both phospholipid and porphyrin contents in the rat Harderian gland, and have shown that HCB affects both metabolisms. Moreover, our results show that the disturbances elicited by HCB in the rat Harderian gland are also strain dependent.

Acknowledgements

We thank Dr A.S. Haedo for help and advice with statistical evaluations and Mrs C. Aldonatti for technical assistance. This work was supported by grants from the National Research Council of Argentina (CONICET), ANPCyT and the University of Buenos Aires.

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Toxicology Letters 106 (1999) 137–141

Toxicology Letters

Prevention of chromosomal aberration in mouse bone marrow by indole-3-carbinol

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Received 29 October 1998; received in revised form 4 February 1999; accepted 10 February 1999

Abstract

In this study, we report the protective effect of indole-3-carbinol (I_3C), one of the glucobrassicin derivative isolated from cruciferous vegetables against cyclophosphamide induced chromosomal aberrations in mouse bone marrow cells. The three test doses namely 1000,500 and 250 mg/kg b.wt. of I_3C provided protection when given 48 h prior to the single i.p. administration of cyclophosphamide (50 mg/kg). I_3C alone did not induce chromosomal aberrations at the test doses of 1000 and 500 mg/kg b.wt.. Thus tested glucobrassicin derivative seems to have a preventive potential against cyclophosphamide induced chromosomal aberrations in Swiss mouse bone marrow cells at the doses tested. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Mutagenicity; Indole-3-carbinol; Cyclophosphamide; Bone marrow; Chromosomal aberrations

1. Introduction

Consumption of cruciferous vegetables is associated with a decreased incidence of cancer in humans (Graham, 1983; Hirayama, 1986). The anticarcinogenic properties of cruciferous vegetables and isolated compounds have been reported in several investigations (Stoewsand et al. 1978; Boyd et al. 1982; Wattenberg, 1983). The hydrolysis product of glucobrassicin viz. indole-3-carbinol, indole-3-acetonitrile and 3-3 dienolylmethane are thus known to inhibit the benzo(a)pyrene induced forestomach tumorigenesis (Loub et al., 1975). The naturally occurring indoles also reduce the benzo(a)pyrene induced sister chromatid exchange (SCE's) frequencies in vitro in primary chick embryo hepatocytes (Jongen et al., 1989). I_3C also inhibited the formation of colonic aberrant crypt foci induced by the hetrocyclic amines (Guo et al., 1995; Xu et al., 1996). Conversely, the tumour promoting effect of

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PII: S0378-4274(99)00060-0

I_3C in colon, liver and thyroid gland are also reported in rat multi organ carcinogenesis test system (Dashwood et al., 1991; Kim et al., 1997; Pence et al., 1986). Indole-3-carbinol induced ornithine dicarboxylase (ODC) activity in mouse epidermis and thus presented potential for enhancement of tumour promotion (Birt et al., 1986). Among various indoles, I_3C has received special attention as a possible chemopreventive agent (Verhoeven et al., 1997). Studies reporting the chemopreventive effect of I_3C in humans are also available in the literature (Anon., 1996; Michnovicz et al., 1997). Nevertheless studies reporting the preventive role of indole-3-carbinol against the mutagenic changes and chemical carcinogenesis are limited and are of considerable importance with respect to nutrition and cancer. Therefore we undertook the evaluation of the preventive effect of I_3C using the chromosomal aberration assay in bone marrow of Swiss mice.

2. Materials and methods

Indole-3-carbinol and cyclophosphamide were purchased from Sigma Chemical Co., St. Louis, MO, USA. Other Reagent grade chemicals were procured locally. The male Swiss albino mice of 8–10 weeks old and 20–25 g b.wt. were obtained from the animal colony of Industrial Toxicology Research Centre, Lucknow. Animals were housed in plastic cages and provided standard pellet diet and water ad libitum.

For the chromosomal aberration assay, the requisite dose of I_3C was dissolved in 5 ml dimethyl sulfoxide (DMSO) and administered as single dose of 0.2 ml per mouse in the group of six animals i.p. 48 h prior to the cyclophosphamide administration. Control mice were injected with an equal volume of vehicle alone. The positive control group also received a single i.p. injection of 50 mg/kg b.wt. dose of cyclophosphamide in 0.9% saline. The animals were sacrificed by cervical dislocation, 24 h after the treatment. Colchicine (4 mg/kg b.wt) was administered intraperitoneally 2 h before killing the animals. The slides were prepared essentially as per modified method (Preston et al., 1987). Femur bones were excised and the bone marrow extracted in 0.56% KCl. In brief the harvested cells were incubated at 37°C for 20 min and then centrifuged for 10 min at 1000 rpm. Cells were fixed in Carnoy's fixative (methanol:acetic acid, 3:1) and burst opened on a clean slide to release chromosomes. The slides stained with 5% Giemsa solution for 15 min and then put in xylene and mounted with DPX.

A total of 100 well spread metaphase plates were scored for chromosomal aberration at a magnification of $1000 \times (100 \times 10 \times)$ for each group. Chromatid breaks, gaps, fragmentation, polyploidy and centromeric association, etc., were scored to assess the chromosomal aberrations. The data are expressed as % chromosomal aberrations. The mitotic index of each group was calculated as per the following formula and the % inhibition by I_3C was calculated against the cyclophosphamide treated group.

$$\text{Mitotic index \% (M.I.)} = \frac{\text{The number of dividing cells}}{\text{Total number of bone marrow cells counted}} \times 100 \quad (1)$$

Statistical significance was calculated using Student's t-test at $P < 0.05$.

3. Results

I_3C administered i.p. at the dose of 1000, 500 and 250 mg/kg b.wt. provided protection against cyclophosphamide induced chromosomal aberrations (Table 1). The degree of protection was 52, 28 and 19%, respectively. A statistically significant protection was observed only with 1000 mg/kg dose levels. All kinds of observed aberrations viz. breaks, gaps, fragmentation's, polyploidy and centromeric associations were found to be protected.

In the positive control group, cyclophosphamide induced different types of the chromosomal aberrations at the dose level tested. I_3C alone did not induce the significant increase in frequency of any of these aberrations at the test dose level and was found to be non-cytotoxic since it

Table 1
Protection against cyclophosphamide induced chromosomal aberrations by indole-3-carbinol

Treatment[a]	Mitotic Index	% Chromosomal aberrations	Chromatid break/gap	Chromatid Fragmentation	Polyploidy	Centromeric Association	% Inhibition[c]
Cyclophosphamide (50 mg/kg)	4.05	59.00 ± 10.46*	6	14	4	4	
I_3C + Cyclophosphamide [b]							
a. 1000 mg/kg + 50 mg/kg	6.4	28.37 ± 5.62**	4	8		2	52%
b. 500 mg/kg + 50 mg/kg	7.6	42.50 ± 8.29	9	9	2	2	28%
c. 250 mg/kg + 50 mg/kg	7.2	48.00 ± 10.9	5	13	5	1	19%
I_3C							
a. 1000 mg/kg	7.5	18.00 ± 5.0	5	2			
b. 500 mg/kg	7.4	16.00 ± 4.69	3	3			
Solvent Control (DMSO)	6.1	18.00 ± 10.9	5	3			

[a] Each group consist of six animals.
[b] I_3C was administered 48 h prior to the cyclophosphamide or vehicle administration.
** Significant protection against cyclophosphamide at $P<0.05$
* Significant induction against the solvent control at $P<0.05$
[c] % protection against the cyclophosphamide treated group (positive control).

did not influence the Mitotic index. The test glucobrassicin derivative provided an effective protection against cyclophosphamide induced cell damage.

4. Discussion

The present study revealed that a single i.p. administration of I_3C resulted in a dose dependent inhibition of chromosomal aberrations induced by cyclophosphamide in mouse bone marrow cells when administered 48 h before cyclophosphamide treatment. Its effectivity was observed at clastogenic dose levels of cyclophosphamide, whereas I_3C alone did not induce a significant number of chromosomal aberrations against the solvent control group. The non mutagenic effect of I_3C alone has been observed also in *Salmonella typhimurium* and in Chinese hamster ovary test systems (Kuo et al., 1992; Takahashi et al., 1995). The antimutagenic effect of I_3C has been noticed in a micronucleus assay by administering 500 and 250 mg/kg b.wt. dose of I_3C 24 and 48 h before the cyclophosphamide administration (Agrawal and Mehrotra, 1997; Agrawal and Kumar, 1998). The anticarcinogenic effect of I_3C has also been reported in polycyclic aromatic hydrocarbon (PAH) induced tumorigenesis in rats (Wattenberg and Loub, 1978). All these reports indicate a protective effect of the glucobrassicin derivative. The exact mechanism of protection is however unknown but I_3C and its metabolites have been shown to be able to participate in the various mechanism of chemoprevention by virtue of acting as nucleophile and antioxidants (Shertzer et al., 1988b).Several mechanisms may contribute to protection such as scavenging of potentially toxic electrophiles and free radicals and modification of enzyme profiles that could inhibit and enhance detoxification pathways. I_3C induces phase I and phase II biotransformation reactions viz. cytochrome P_{450} and glutathione-S-transferase activities (Shertzer and Sainspury, 1991; Baldwin and Leblanc, 1992; WorterBoer et al., 1992). Induction of these enzymes which constitute Phase I and II activity of the mixed function oxidase function for metabolism and disposition of xenobiotics may play a significant role in the observed antimutagenic effects of I_3C. The amount of indole-3-carbinol present in cruciferous vegetables is reportedly in the range of 410–1090 mg/kg fruit weight (Fenwick and Heany, 1983). When it is assumed that a person on a vegetarian diet consumes about 100 g cruciferous vegetables per day, the daily intake of I_3C may fall in the range of 41–109 mg per person. Therefore, the dose used is much higher than the actual consumption in humans. It is noteworthy that the employed dose is effective against a noted mutagen. These results support the preventive properties of diet and nutrition in chemical carcinogenesis.

Acknowledgements

Authors are thankful to Dr P.K. Seth, Director of ITRC for his encouragement and for providing the facilities to carry out this work.

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Toxicology Letters 106 (1999) 143–150

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The UDP-glucuronyltransferase inducers, phenobarbital and pregnenolone-16α-carbonitrile, enhance thyroid-follicular cell apoptosis: association with TGF-β_1 expression

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Received 19 October 1998; received in revised form 8 February 1999; accepted 15 February 1999

Abstract

Exposure to certain UDP-glucuronosyltransferase (UDP-GT) inducers leads to follicular cell hyperplasia, and ultimately thyroid gland tumors. These compounds decrease thyroid hormones, which increases serum concentrations of thyroid stimulating hormone (TSH). This induction of TSH enhances thyroid-follicular cell proliferation. In addition, treatment with classical goitrogenic compounds, such as propylthiouracil (PTU) and methimazole (MMI), induces TGF-β_1 in thyroid-follicular cells, presumably through increased TSH. In other tissues, increases in TGF-β_1 induce apoptosis, a particular form of programmed cell death. In this experiment, we sought to determine whether the UDP-GT inducers, phenobarbital (PB) and pregnenolone-16α-carbonitrile (PCN) modulate thyroid-follicular cell apoptosis. If so, are the induction of apoptosis and TGF-β_1 possibly linked? An additional group of rats treated with the thyroid goitrogen, PTU was included. Male Sprague–Dawley rats were treated with thyroid hormone disrupting doses of PB, PCN, or PTU for 3, 7, 14, 21, 28, 45, or 90 days. In this study, PTU treatment increased apoptosis and TGF-β_1 immunoreactive thyroid-follicular cells. PTU treatment of rats produced both a large increase number of TGF-β_1-positive cells (detected by immunohistochemistry), and apoptotic thyroid-follicular cells (detected by morphology). In PB- and PCN-treated rats, a moderate increase in apoptosis coincided with similar increases in TGF-β_1 immunoreactive thyroid-follicular cells. In summary, PB and PCN increase apoptosis and the percentage of TGF-β_1 positive thyroid-follicular cells. Thus, treatment with UDP-GT-inducing chemicals may increase the expression of TGF-β_1 and apoptosis in the thyroid to compensate for the thyroid hypertrophy and hyperplasia. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Apoptosis; TGF-β_1; Thyroid; UDP-GT inducers

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PII: S0378-4274(99)00023-5

1. Introduction

Certain compounds that induce UDP-glucuronosyltransferase (UDP-GT) activity also produce thyroid-follicular cell hyperplasia and promote thyroid tumors (Hill et al., 1989). UDP-GT inducers produce these thyroid pathologies by reducing serum thyroid hormones, leading to feedback induction of thyrotropin (TSH) secretion (Hill et al., 1989). Because thyroid hormones are tightly regulated by the hypothalamus–pituitary–thyroid axis, reduced serum thyroid hormone levels increases the release of TSH (Hill et al., 1989). TSH stimulates the thyroid gland to increase thyroid hormone synthesis and secretion, as well as produce follicular cell hyperplasia and hypertrophy. Indeed, treatment of rats with the UDP-GT inducers, PB and PCN, as well as the thyroid goitrogen, propylthiouracil (PTU), decreases serum levels of thyroid hormones, increases serum levels of TSH, and increases thyroid-follicular cell proliferation leading to an increase in thyroid weight (Barter and Klaassen, 1994; Hood et al., 1995; Liu et al., 1995). Table 1 summarizes the effect of PTU, PB, and PCN on T_3, T_4, TSH, thyroid-follicular cell proliferation, and thyroid weight.

Table 1
Summary of the effects of 30 mg propylthiouracil/kg of diet (PTU), 500 mg phenobarbital/kg of diet (PB) and 500 mg pregnenolone-16α-carbonitrile/kg of diet (PCN) on thyroid hormones, TSH, thyroid proliferation, and thyroid weight[a]

	UDP-GT inducer		
	PB	PCN	PTU
Serum-free T_4	↓	↓↓	↓↓↓↓
Serum-free T_3	–	–	↓↓
Serum TSH	↑	↑↑	↑↑↑↑
Thyroid-follicular cell proliferation	↑	↑↑	↑↑↑
Thyroid weight	↑	↑↑	↑↑↑

[a] Arrows indicate the relative change due to treatment when compared to control. There is a pronounced effect of PTU on all thyroid parameters examined when compared from rats treated with the UDP-GT inducing agents, PB and PCN.

TSH induces transcription and secretion of TGF-β_1, a 25-kDa peptide that is a potent growth inhibitor of many epithelial cell types, including thyroid-follicular cells (for a review see Sporn and Roberts, 1985; Koli and Keski-Oja, 1996) TSH increases TGF-β_1 mRNA and protein secretion in a rat thyroid cell line, FRTL-5 (Pekary et al., 1995). PTU and methimazole (MMI) treatments, which are both known to increase TSH (Oren et al., 1995; Morosini et al., 1996) can induce thyroid-follicular cell TGF-β_1 mRNA and protein (Logan et al., 1994; Morosini et al., 1996). Once stimulated, TGF-β_1 can stimulate further TGF-β_1 production (Border and Ruoslahti, 1992). The paradoxical increase in expression of TGF-β_1 in hyperplastic thyroid may help blunt the proliferative stimulus of TSH and stabilize thyroid goitre growth and involution (Logan et al., 1994; Morosini et al., 1996).

TGF-β_1 is not only an inhibitor of cell replication, but also may mediate apoptosis, a form of programmed cell death (Oberhammer et al., 1993). In hepatocytes, TGF-β_1 receptor activation has been suggested to be the stimulus for apoptotic cell death (Oberhammer et al., 1993). In cultured porcine thyroid-follicular cells, TGF-β_1 induces apoptosis (Froschl et al., 1994). In a human medullary thyroid carcinoma cell line, TGF-β_1 treatment decreased cell proliferation and increased the rate of cell death (Khosla et al., 1994). However, the ability of TGF-β to induce apoptosis may not be so clear in vitro. In FRTL-5 cells, TGF-β did not induce apoptosis (Pekary et al., 1995) or did so only in quiescent cells (Carneiro et al., 1998). In any case, thyroid-follicular cell loss of responsiveness to TGF-β_1 growth inhibition may represent a critical event on the path to thyroid tumorigenicity (Blaydes and Wynford-Thomas, 1996).

In this study, we hypothesize that the UDP-GT inducers, PB and PCN, which have been shown to increase serum TSH (Barter and Klaassen, 1994; Liu et al., 1995) will also increase TGF-β_1 expression in the thyroid. An increase in TGF-β_1, we further hypothesize, will induce apoptosis. This study examined the effect of UDP-GT-inducing agents on apoptosis and TGF-β_1 induction in thyroid-follicular cells.

2. Materials and methods

2.1. Chemicals

Propylthiouracil was purchased from Sigma (St. Louis, MO). Phenobarbital was purchased from Spectrum Chemical (Gardena, CA). Pregnenolone-16α-carbonitrile was prepared from 16-dehydropregnenolone (Pfaltaz & Bauer, Watbury, CT) as described previously (Sonderfan and Parkinson, 1988).

2.2. Animals and experimental design

One hundred and fifty pathogen-free male Sprague–Dawley rats, aged-matched (between 225 and 250 g) (Sasco, Omaha, NE) were divided randomly into groups of five to six rats, housed in polypropylene cages, and maintained at 70°F on a 12-h light/dark cycle. Throughout the entire study, all rats were maintained in an AAALAC-accredited animal facility according to the NIH-Guide for the Care and Use of Laboratory Animals. During a 1-week acclimation period, rats were administered tap water and standard laboratory rodent chow (Ralston-Purina, Chicago, IL) ad libitum. After the acclimation period, rats were randomly separated into one of the four following treatment groups (doses selected based on previous studies that have shown thyroid hormone disruption):

Group no.	Group name	Treatment
1	Control	Control diet
2	PTU	30 ppm propylthiouracil
3	PCN	500 ppm pregnenolone-16α-carbonitrile
4	PB	500 ppm phenobarbital

Five rats per group per time point were euthanized after 3, 7, 14, 21, 30, 45, 60 and 90 days of dietary treatment, except in Group 1 (control). These time points were selected to characterize the response to thyroid hormone response at pre- and post-compensatory times. Data from rats in the control group euthanized at day 3 represent both days 3 and 7, and data from rats euthanized at day 14 represent both days 14 and 21. Rats were asphyxiated by CO_2, weighed and necropsied. Thyroid glands were removed, weighed, formalin-fixed (minimum of 24 h), paraffin-embedded, sectioned (5 μm), and mounted on glass slides for histology.

2.3. Diet preparation

Test articles were dissolved in acetone and applied to known amount of diet to achieve the desired concentration. The diet was continually shaken until the acetone had dried. Diet containing PCN, PB, and PTU was prepared as described monthly, stored at approximately −4°C and administered ad libitum. Food consumption was monitored weekly with no significant difference among groups (data not shown).

2.4. Apoptosis

Microscopic evaluation of hematoxylin and eosin-stained slides was used to quantify the incidence of apoptosis. Apoptosis was defined by morphological characteristics similar to those described in other tissues (Bursch et al., 1984, 1985; Kolaja et al., 1996), which includes: (A) increased cytoplasmic eosinophilia; (B) increased nuclear condensation; (C) fragmentation of DNA; and (D) separation from surrounding follicular cells. At least 5000 cells per slide were examined for the histological presence of apoptosis. Apoptotic index was determined by dividing the number of apoptotic follicular cells by the total number of follicular nuclei observed. In addition, the morphological presence of apoptosis was verified by immunohistochemical detection of fragmented DNA (Apoptag® Kit, ONCOR®, Gaithersburg, MD) on a few randomly selected slides.

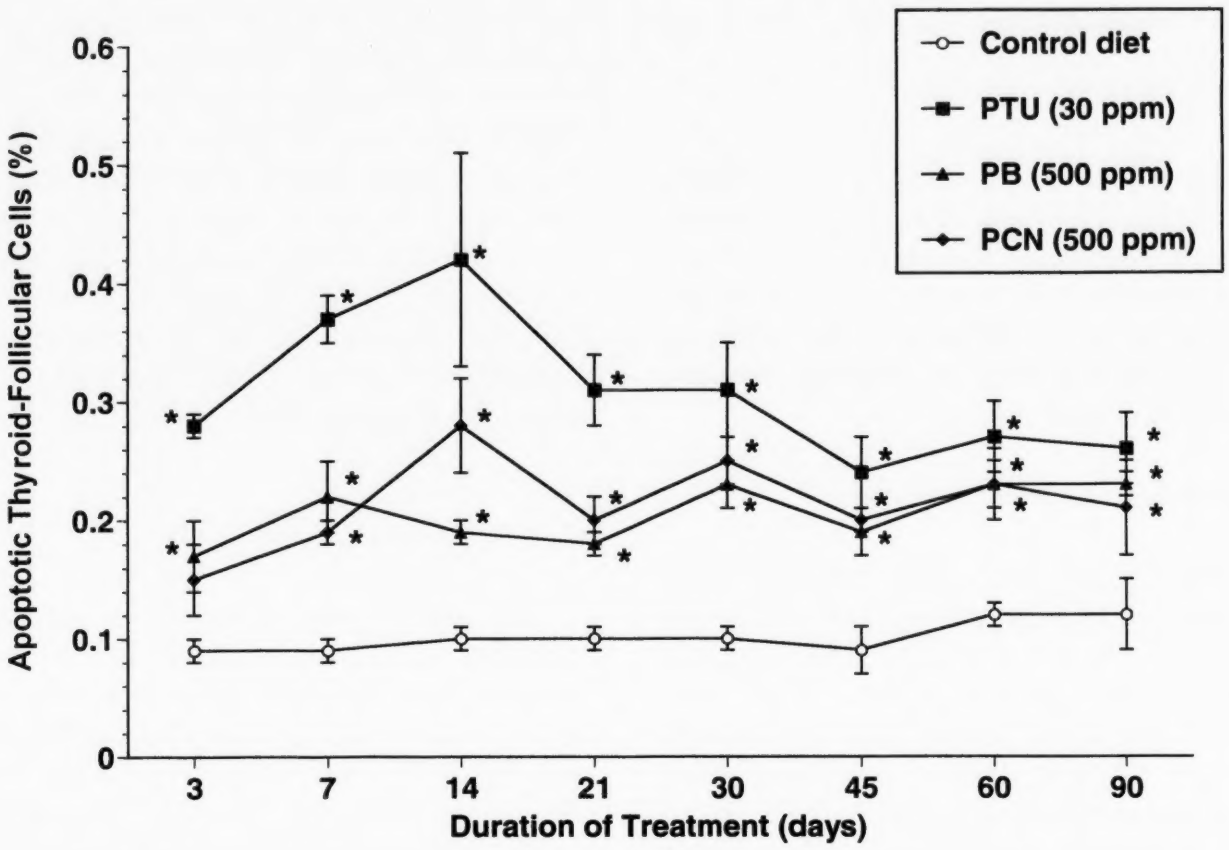

Fig. 1. Incidence of apoptotic thyroid-follicular cells of rats treated with 30 mg PTU/kg of diet, 500 mg PB/kg of diet and 500 mg PCN/kg of diet. Values represent the mean ± the S.E. of five to six rats per group. Statistical significance (*$P < 0.05$) was determined by ANOVA followed by Duncan's post-hoc test.

2.5. TGF-β_1 immunohistochemistry

TGF-β_1 immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue as previously described with minor modifications (Logan et al., 1994). Briefly, slides were incubated with primary antibody (Santa Cruz Biotech., Santa Cruz, CA) for 48 h (4°C) at a dilution of 1:25. An Avidin–biotin complex kit (Santa Cruz Biotech) was used per manufacturers' instructions for secondary, tertiary, and diaminobenzidine chromagen reagents. TGF-β_1 was visualized by the presence of brown pigment in TGF-β-positive cells as compared to the unstained, nonlabeled cells. TGF-β_1 labeling index was determined by dividing the number of follicular cells with TGF-β_1-positive cells by the total number of follicular cells observed. At least 3000 thyroid-follicular cells per slide were scored for the immunohistochemical presence of TGF-β_1.

2.6. Statistics

Statistical significance ($P < 0.05$) from the control group for all data was determined by ANOVA, followed by Duncan's multiple range post-hoc test.

3. Results

3.1. Apoptosis

Apoptosis was detected by morphological criteria (Bursch et al., 1984, 1985; Kolaja et al., 1996) and validated by immunohistochemical detection of fragmented DNA (Kolaja et al., 1996). Thyroids from control, untreated rats had large colloidal spaces, and a low incidence of apoptosis (0.1%) (Fig. 1). In PTU-, PB- and PCN-treated rats, thyroids were hyperplastic, as relatively little

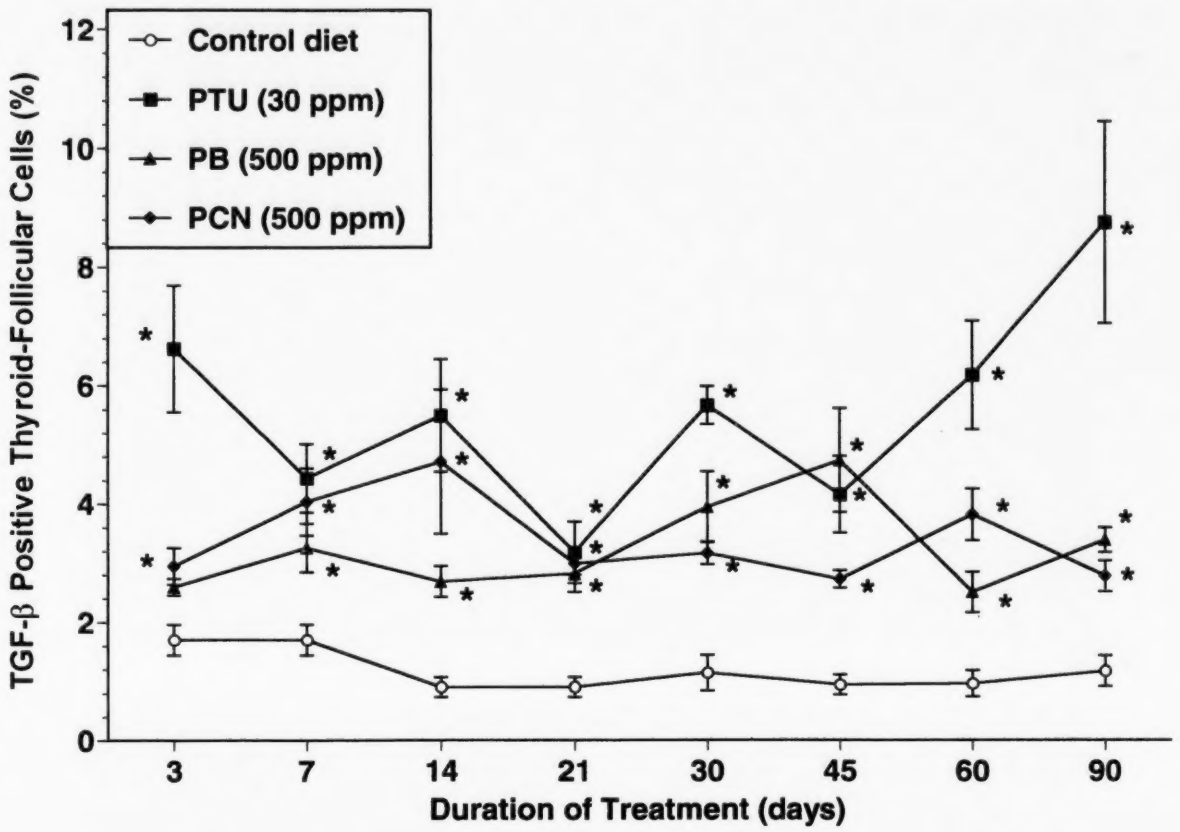

Fig. 2. Immunohistochemical expression of TGF-β_1 in thyroid-follicular cells (percent of total follicular cell population) of rats treated with 30 mg PTU/kg of diet, 500 mg PB/kg of diet and 500 mg PCN/kg of diet. Values represent the mean (the S.E. of five to six rats per group. Statistical significance (*$P < 0.05$) was determined by ANOVA followed by Duncan's post-hoc test.

colloidal space was evident. Interestingly, apoptotic cells were apparent (Fig. 1).

Treatment with PTU, PB, or PCN increased the incidence of apoptosis (Fig. 1). In PTU-treated rats, apoptosis increased 3–4-fold at the earlier time points (3, 7 and 14 days). From 21 to 90 days of treatment, the incidence of apoptosis was roughly 3-fold higher in PTU-treated rats than control rats (Fig. 1). PB treatment of Sprague–Dawley rats increased thyroid-follicular cell apoptosis 2-fold throughout the treatment. In PCN-treated rats, a similar 2-fold increase was observed after 7 days of dietary treatment (Fig. 1).

3.2. TGF-β_1 immunohistochemical expression

The percentage of thyroid-follicular cells that were TGF-β_1 immunoreactive increased in PTU-, PB- and PCN-treated rats (Figs. 2 and 3). TGF-β_1-positive cells increased after 3, 7 and 14 days of treatment with PTU, a time when PTU treatment led to a similar increase in apoptosis. In PB- and PCN-treated rats, the percentage of thyroid-follicular cells expressing TGF-β_1 increased by 50%.

4. Discussion

Treatment with the UDP-GT inducers, PB and PCN, as well as the thyroid goitrogen, PTU, increased the incidence of apoptosis. This increase in programmed cell death was coincident with the immunohistochemical presence of an apoptosis-inducing growth factor, TGF-β_1. Treatment with goitrogenic compounds, such as PTU and the UDP-GT inducers, PB and PCN, all increase thyroid-follicular cell proliferation (Pekary et al., 1995), but, to our knowledge, this is the first indication that goitrogenic compounds also affect apoptosis.

The increase in apoptosis observed in hyperplastic, proliferating thyroid glands appears, at first, to be a contradictory finding. However, the increased proliferation observed in PTU-, PB- and PCN-

(a)

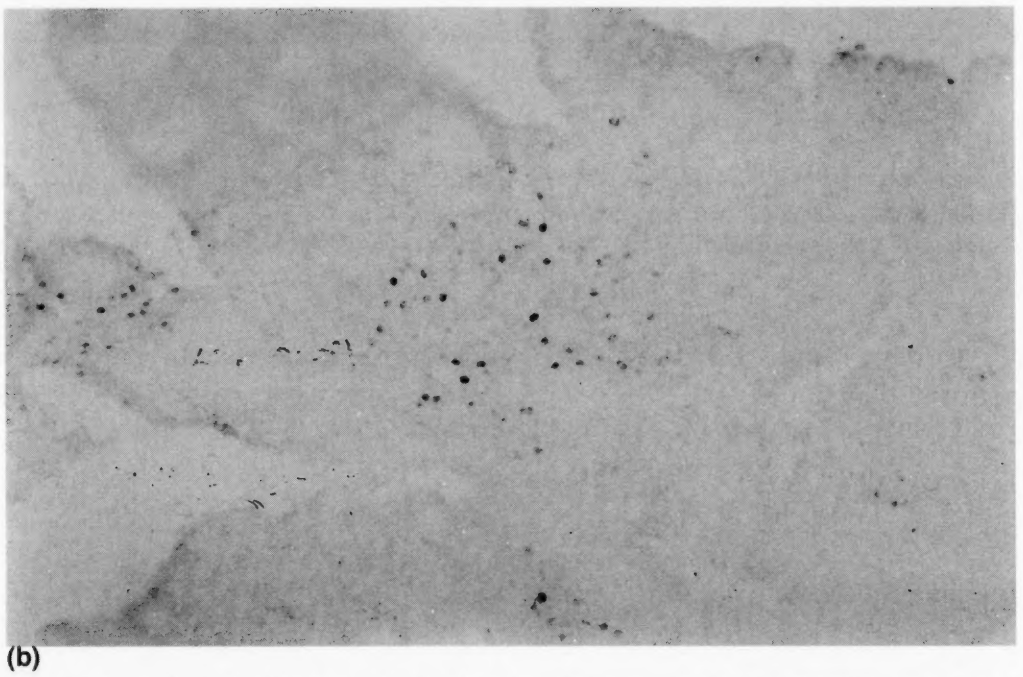
(b)

Fig. 3. Immunohistochemical detection of TGF-β_1 in thyroid sections from control (a) and PTU-treated rats (b). Notice the relative lack of immunopositive cells in the control thyroid (the positive cells are mostly neuronal-derived parafollicular or C cells). In contrast, thyroids from PTU-treated rats (shown is 3 days of treatment) had numerous thyroid-follicular cells which were positive for TGF-β_1 expression.

treated rats is 10-fold greater than the increase in apoptosis (Hood et al., 1995). The induction of apoptosis may remove excess thyroid-follicular cells during this period of increased proliferation. In support of this increased apoptosis during increased cell proliferation, an increase in apoptosis is observed in several thyroid proliferative diseases (Okanayu et al., 1995).

TGF-β_1 can induce apoptosis (Froschl et al., 1994). In several thyroid diseases (e.g. Graves' disease, Hashimoto's disease, thyroiditis, thyroid adenoma, and thyroid carcinoma) where TSH is elevated, thyroid-follicular apoptosis increases (Okanayu et al., 1995). TSH may stimulate this increased cell death by increasing TGF-β_1 (Okanayu et al., 1995). TGF-β_1 treatment induces

apoptosis in cultured thyroid-follicular cells (Froschl et al., 1994). In the present study, TGF-β_1 protein was increased in all three treatment groups (PTU, PB and PCN), and associated with the morphological presence of apoptosis.

Chronic administration of UDP-glucuronosyltransferase inducers can increase the incidence of thyroid neoplasia (Hill et al., 1989). The proposed mechanism of action is the chronic stimulation of the thyroid by TSH. This increased TSH may 'promote' the growth of spontaneously initiated thyroid-follicular cells. TSH-induced TGF-β may assist the thyroid carcinogenic process by either increasing apoptosis leading to an aberrant, exaggerated proliferation of certain preneoplastic thyrocytes, or by enhancing the growth of preneoplastic thyroid-follicular cells that are more resistant to the TGF-β-induced cell death than surrounding cells.

Thyroid-follicular cells can acquire resistance to TGF-β_1-mediated growth inhibition and cell death (Asmis et al., 1996). A small percentage of cultured thyroid-follicular cells (i.e. FRTL-5, several feline thyroid cell lines, and human primary cultures), as well as a large percentage of thyroid cells isolated from goiters, are insensitive to the growth-inhibiting properties of TGF-β_1, thus potentially developing a growth advantage (Pang et al., 1992; Asmis et al., 1996). This type of acquired resistance to TGF-β_1-mediated effects may explain why TGF-β_1-positive cells were not necessarily the thyroid-follicular cells that were also undergoing apoptosis. As the TGF-β_1-producing thyroid-follicular cells increase, cells that are sensitive to TGF-β_1 are eliminated via apoptosis, suggesting a paracrine effect. Thus, it is possible that certain thyroid-follicular cells acquire a growth advantage and they over-express TGF-β_1, yet are less susceptible to apoptosis.

It has been suggested that there are three main roles for TGF-β_1 in the thyroid (Roger, 1996). These include: (1) a direct inhibition of proliferation by antagonizing the activities of TSH and cAMP; (2) a chemotactic, inflammatory response important in tissue wound healing by attracting fibroblasts and enhancing extracellular matrix production (e.g. scarring); and (3) a potent immunosuppressive effect possibly by impeding B and T lymphocyte proliferation and/or preventing MHC class II antigen expression (Kulkarni and Karlsson, 1993), as TGF-β_1 treatment inhibits the development of a variety of experimentally induced autoimmune diseases. We speculate that a fourth role may exist, based on evidence presented in this paper and in the literature, that TGF-β_1 induces apoptosis in thyroid-follicular cells as a protective growth inhibitory mechanism.

In this study, treatment of PTU, PB and PCN increased apoptosis paradoxically during rat thyroid hyperplasia. Coincident with this increased cell death, is an increase in the immunohistochemical detection of TGF-β_1. This increase in TGF-β_1, may aid in inducing thyroid-follicular cell apoptosis.

Acknowledgements

Supported by NIH grant ES-03192 and by the NRSA grant ES-05812 and the Kansas Health Foundation.

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Toxicology Letters 106 (1999) 151–157

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Glutathione depletion and oxidative damage in mitochondria following exposure to cadmium in rat liver and kidney

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Received 3 June 1998; received in revised form 11 February 1999; accepted 22 February 1999

Abstract

The dose-dependent effects of cadmium (Cd) on mitochondria and post-mitochondrial supernatant (PMS) of liver and kidney were investigated in adult male albino rats. Two groups of rats were injected intraperitoneally with 0.1 mg Cd/kg body weight and 1 mg/kg body weight, respectively, for a period of 3 months (5 days/week). This resulted in a significant decrease in total glutathione (GSH) levels, irrespective of the doses, in mitochondrial as well as in PMS fractions of liver and kidney. In contrast, end products of lipid and protein were significantly increased in a dose-dependent manner in subcellular fractions of liver and kidney. These results suggest that the depletion of tissue glutathione levels is not a primary reason of the observed oxidative damage in liver and kidney caused by Cd. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cadmium; Mitochondria; Post-mitochondrial fraction; Reduced glutathione; Lipid peroxidation; Protein carbonyls

1. Introduction

With increasing production and utilisation of cadmium, not only industrial workers but the general population is also exposed to the toxic effects of Cd. This has been found to produce various pathological conditions like hepatic and renal dysfunctions, testicular damage, respiratory and nervous system disorders (Underwood, 1977). Cd produces oxidative stress by disturbing the prooxidant–antioxidant balance, in favour of the former (Sies, 1985) because it has very high affinity for the sulphydryl groups in reduced glutathione (GSH) which may have implications in the maintenance of the thiol–disulphide balance in the cell (Ziegler, 1985) and its organelles. Although numerous data are available concerning the effect of Cd on whole tissue of various vital organs (Hussain et al., 1987; Iguchi et al., 1991;

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PII: S0378-4274(99)00059-4

Manca et al., 1991; Leelank and Bansal, 1996; Kumar et al., 1996), little is known about the possible involvement of GSH and its correlation with protein and lipid oxidation in the Cd-exposed mitochondria.

Mitochondria are known to be vital organelles for supporting the energy-dependent regulation of various cell functions. Interference with mitochondrial respiration results in profound bioenergetic deficits leading to the loss of various vital functions of the cell which are indispensable for its survival and ultimately, for the organisms Wallace et al., 1997. Even under well-coupled conditions, very little amount of mitochondrial O_2 consumption generates superoxide anion free radicals and H_2O_2 during aerobic metabolism which will result in the formation of lipid hydroper oxides that can damage mitochondrial membranes and their functions. Furthermore, injury to mitochondrial membrane is a crucial factor in the various oxidative stress-mediated cell death (Massini et al., 1985) Therefore, mitochondrial GSH (mt-GSH) could play a critical role in the defense against membrane peroxidation and also in regulation of inner membrane permeability (Kosower and Kosower, 1983; Beatrice et al., 1984). Wallace et al. (1997) have shown that mitochondria contribute more to cell sustenance than ATP synthesis. It has been reported earlier that depletion of mt-GSH was found to be time- and dose-dependent, which is somewhat slower than the loss of cytoplasm GSH (Meredith and Reed, 1982) because decrease in mt-GSH to about one half of control level is responsible for the loss of cell viability (Olafsdottir et al., 1988). We have demonstrated that 1 day exposure of Cd (0.1 and 1.0 mg/kg;i.p.) could not able to cause GSH depletion and increase in oxidatively damaged end products in mitochondrial and PMS fractions of liver and kidney (unpublished). Manca et al. (1991) have also examined the same in whole tissues of various vital organs. Keeping in view, we have selected a low-dose of Cd (0.1 mg/kg) for long-term exposure along with a 1 mg Cd/kg dose to see whether there is dose-dependent effect of Cd on glutathione depletion and oxidatively damaged end products in mitochondrial and PMS fractions of liver and kidney.

2. Materials and methods

2.1. Chemicals

Reduced glutathione and *o*-pthalaldehyde were purchased from Sigma Chemical Company, St. Louis, MO, USA. Cadmium chloride and other routine chemicals were of AnalR grade and were obtained from Sisco Research Laboratory, Bombay, India.

2.2. Animals and treatment

Male albino rats of Druckrey strain obtained from Industrial Toxicology Research Centre breeding colony weighing 100 ± 10 g were divided at random into three groups (15 animals per group). The animals were kept in stainless steel cages in normal laboratory conditions where 12-h light/dark cycle was maintained. Animals had free access to drinking water and pellet diet obtained from Hindustan Lever Laboratory Feed, Calcutta, India. Group II and group III rats were administered with Cd as $CdCl_2$ at a dose of 0.1 mg/kg and 1 mg/kg body weight intraperitoneally (i.p.) in 0.5-ml physiological saline, respectively, for 3 months (5 days/week). Group I animals were treated with 0.5-ml physiological saline in an identical manner for the same period.

2.3. Sample preparation

At 24 h after the last injection, six animals from each group were sacrificed by decapitation. Liver and kidney were removed, weighed and homogenized (10%) immediately in chilled 0.25 M sucrose solution and mitochondrial and PMS fractions were prepared according to the procedure of Estrabrook and Pullman (1967)).

2.4. Oxidatively damaged end products

Lipid hydroperoxides (LH) were measured by the method of Buege and Aust (1978). The colour was measured spectrophotometrically at 532 nm. Results were presented in arbitrary absorbance U/g tissue.

Conjugated dienes (CD) were measured according to Ohakawa et al. (1976). The absorbance was read against a *n*-heptane blank at 233 nm. Results were presented in arbitrary absorbance units/g tissue.

The levels of protein carbonyls were measured according to the procedure of Levin et al. (1990). Samples were read at 366 nm against guanidine hydrochloride blank. Protein carbonyl levels were expressed as nmol/mg protein.

2.5. Reduced glutathione

Reduced glutathione (GSH) was estimated according to the method of Hissin and Hilf (1976) using *o*-pthalaldehyde. Samples were measured flourometrically (excitation 350 nm/emission 420 nm). GSH levels were presented as nmol/g tissue.

2.6. Protein determination

Protein estimation was done by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.7. Statistical procedure

The data were analyzed using one-way ANOVA and the significance of the difference between two groups was evaluated by student's *t*-test. $P < 0.05$ were considered to be significant.

3. Results

Body weight of treated animals was found to be significantly decreased by 16% at low dose and 25% at high dose as compared to control rats.

Table 1 shows the dose-dependent effects of cadmium on lipid peroxidation. The levels of conjugated dienes in liver mitochondria were increased by 49% at 0.1 mg/kg dose and 79% at 1.0 mg/kg dose. Conjugated diene levels were altered in kidney mitochondria also, showing an increase of 47% at low dose and 71% at high dose. Conjugated dienes in PMS fraction of kidney were increased by 23% and 73% at low and high doses, respectively. In liver PMS supernatant conjugated diene levels were also increased significantly at low dose (31%) and high dose (59%) as compared to vehicle treated controls. Lipid hyroperoxide levels in liver PMS fraction were increased by 27% at low dose and 60% at high dose. However, mitochondrial levels were increased (21%) at high dose only. In post-mitochondrial supernatant of kidney lipid hydroperoxides were increased by 17% and 30% at low and high doses, respectively. Lipid hydroperoxide levels in mitochondrial frac-

Table 1
Effect of cadmium treatment on oxidative end products of lipids[a]

Parameters	Group	Liver		Kidney	
		PMS	Mit	PMS	Mit
Conjugated	I	3.63 ± 0.26	0.89 ± 0.06	2.30 ± 0.17	0.86 ± 0.06
Dienes (U/g tissue)	II	4.76 ± 0.34^{b}	1.12 ± 0.07^{b}	2.83 ± 0.15^{b}	1.26 ± 0.09^{c}
	III	5.76 ± 0.28^{d}	1.58 ± 0.11^{d}	4.0 ± 0.28^{d}	1.47 ± 0.10^{d}
Lipid hydroperoxide (U/g tissue)	I	5.86 ± 0.32	2.09 ± 0.11	4.96 ± 0.22	2.82 ± 0.16
	II	7.44 ± 0.57^{b}	2.35 ± 0.12	5.80 ± 0.28^{b}	2.86 ± 0.15
	III	9.38 ± 0.86^{c}	2.53 ± 0.15	6.47 ± 0.34^{c}	3.42 ± 0.19^{b}

[a] Value represent mean ± SE of five samples (animals per group). Value in the parentheses are percent change compared to their corresponding controls. PMS, Postmitochondria supernatant, Mit, Mitochondrial fraction; GrI, Control; GrII, Cd; 0.1 mg/kg b.wt., GrIII, 1.0 mg/kg b.wt.

[b] $P<0.05$.

[c] $P<0.01$.

[d] $P<0.001$ (Student's *t*-test).

Table 2
Effect of cadmium treatment on the levels of protein carbonyl[a]

Parameters	Groups	Liver		Kidney	
		PMS	Mit	PMS	Mit
Protein carbonyls (nmol/mg)	I	27.3 ± 1.51	20.5 ± 1.19	17.5 ± 1.18	14.2 ± 0.91
	II	30.5 ± 2.56	20.9 ± 1.20	17.6 ± 1.21	15.5 ± 1.01
	III	50.5 ± 4.22[d]	24.5 ± 1.22[b]	23.9 ± 1.22[c]	17.3 ± 0.98[b]

[a] Value represent mean ± SE of five samples (animals per group). Value in the parentheses are percent change compared to their corresponding controls. PMS, Postmitochondria supernatant, Mit, Mitochondrial fraction; GrI, Control; GrII, Cd; 0.1 mg/kg b.wt., GrIII, 1.0 mg/kg b.wt.
[b] $P<0.05$.
[c] $P<0.01$.
[d] $P<0.001$ (Student's t-test).

tion of kidney did not alter at 0.1 mg dose; however, it increased significantly (21%) at 1.0 mg dose as compared to control rats.

The administration of cadmium at low dose did not change protein carbonyl contents in liver and kidney. However, Cd at 1 mg/kg dose increased its contents by 72 and 37% in PMS fractions of liver and kidney, respectively. Mitochondrial protein carbonyl contents at high Cd dose were increased by 20 and 21%, respectively, in liver and kidney (Table 2).

The data in Table 3 show the dose-dependent effect of Cd on the levels of glutathione and protein content in cellular fractions of liver and kidney. Cadmium decreased the levels of glutathione in the mitochondria of liver by 19% and 25% at low and high doses, respectively, whereas glutathione levels were decreased by 18% at low dose and 23% at high dose in kidney mitochondria. Cadmium administration decreased the levels of glutathione significantly in PMS fraction by 22–31% in liver and 19–27% in kidney at low and high doses, respectively, as compared to vehicle treated controls. Protein levels were decreased slightly in both the fractions of liver and kidney in the case of high dose. At low dose there was no change in mitochondrial fractions of both the organs, whereas slight decrease in PMS fractions of liver and kidney.

4. Discussion

In the present study, animals subjected to cadmium exposure showed a significant decrease in glutathione levels, irrespective of the doses, in mitochondrial as well as in PMS fractions of liver and kidney. The decrease in the GSH level may be due to the increased activity of γ-glutamyl transpeptidase enzyme as also suggested by Karmakar et al. (1998) following Cd exposure in liver and kidney tissues. Another possible reason for decreased GSH levels may be due to increased levels of lipid oxidation products which could be associated with the less availability of NADPH required for the activity of glutathione reductase to transform oxidised glutathione(GSSG) to the reduced form (GSH) (Sarkar et al., 1995). Chin and Templeton (1993) have demonstrated that cadmium produced dose- and time-dependent increases in intracellular glutathione concentrations. However, at high Cd concentrations, the glutathione depletion that occurs may be due to the production of reactive oxygen species at a rate that exceeds the ability to regenerate reduced glutathione. Our results are in accord with this study in the case of high dose of Cd. However, decreased levels of glutathione at low dose in both the cellular fractions also, may be due to the difference in the dose, route and long-term exposure to cadmium.

Recently, we have demonstrated that single exposure to Cd (0.1 and 1.0 mg/kg; i.p.) did not alter the glutathione levels in both mitochondrial and PMS fractions of liver and kidney (unpublished). Earlier it has been reported that a continuous intake of Cd tends to accumulate in the body with time because there is no effective homeostatic mechanism present in the body to deal with the constant exposure to Cd (Nriagu, 1981). Our recent studies (unpublished) have also shown decreased levels of glutathione in mitochondria as well as in PMS of brain following i.p. administration of 0.4 mg Cd/kg to adult rats for 30 days (5 days/week).

GSH depletion decreases the GSH/GSSG ratio which leads to the production of free radicals (Leelank and Bansal, 1996). These free radicals interact with proteins and membrane lipids leading to the production of protein carbonyls in addition to modifications of amino acids (Ursini et al., 1991) and lipid hydroperoxides (Davies, 1987), respectively. Cd treatment in the present experiment shows a significant increase in the levels of protein and lipid oxidation products in a dose-dependent manner suggests that glutathione depletion may not be a primary reason for increased oxidatively damaged end products. Hussain et al. (1987) have also reported that whether the increase in lipid peroxidation occurs as a direct effect of Cd or a decrease in glutathione content, is not clear. Cd intoxication also increases the iron content in addition to lipid peroxide levels which may be due to iron displacement, resulting in enhanced iron-mediated lipid peroxidation (Koizumi and Li 1992; Casalino et al., 1997). The elevated lipid peroxide levels lead to the significant changes in the membrane functions including collapse in transmembrane potential, release of intramitochondrial calcium, uncoupling and mitochondrial swelling (Vladimirov et al., 1980; Massini et al., 1985; Bindoli, 1988; Castillo et al., 1994). Kumar et al. (1996) have also reported the same. Earlier, it has been proposed that increased membrane lipid rigidity (Dobretsov et al., 1977; Vladimirov et al., 1980) and negative surface charges (Vladimirov et al., 1980) during lipid peroxidation may form peroxide clusters in isolated mitochondria and acts as pores across the membrane (Augustin et al., 1979; Bindoli, 1988). Moreover, decomposition products of lipid hydroperoxides such as malonaldialdehyde and 4-hydroxynonenal (Halliwell and Gutteridge, 1989) may also contribute to the damage by forming schiff bases with membrane proteins (Halliwell and Gutteridge, 1989; Castillo et al., 1994) and thus destabilising the membrane structure.

In summary, the results suggest that Cd-induced elevation in oxidatively damaged end products may not be only due to the possibility of lower level of glutathione but could also be as a result of direct action of Cd on peroxidation reaction/iron-mediated peroxidation.

Table 3
Effect of cadmium treatment on the levels of reduced glutathione (GSH) and protein contents[a]

Parameters	Groups	Liver		Kidney	
		PMS	Mit	PMS	Mit
GSH (μmol/g)	I	71.0 ± 4.26	11.2 ± 0.86	67.6 ± 4.36	10.5 ± 0.64
	II	55.2 ± 3.38[b]	9.16 ± 0.25[b]	54.6 ± 3.48[b]	8.63 ± 0.38
	III	49.0 ± 2.01[c]	8.4 ± 0.30[b]	49.1 ± 2.96[b]	8.1 ± 0.40[b]
Protein (mg/g)	I	23.8 ± 1.32	3.44 0.19	23.7 1.51	3.61 0.24
	II	20.4 ± 1.19	3.04 0.16	21.8 1.46	3.52 0.25
	III	19.8 ± 1.18[b]	2.80 ± 0.18[b]	20.2 ± 1.12[b]	2.88 ± 0.14[b]

[a] Value represent mean ± SE of five samples (animals per group). Value in the parentheses are percent change compared to their corresponding controls. PMS, Postmitochondria supernatant, Mit, Mitochondrial fraction; GrI, Control; GrII, Cd; 0.1 mg/kg b.wt., GrIII, 1.0 mg/k b.wt.

[b] $P < 0.05$.

[c] $P < 0.01$. (Student's t-test).

Acknowledgements

The authors are grateful to Dr P.K. Seth, Director, Industrial Toxicology Research Centre, Lucknow for his keen interest and valuable suggestions. The Council of Scientific and Industrial Research (CSIR), New Delhi, is also acknowledged for providing fellowship to Ms Darshika Nigam under C.S.I.R. Young Scientist Award to GSS.

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Toxicology Letters 106 (1999) 159–169

Toxicology Letters

Hepatic cytochrome P450 and flavin-containing monooxygenase in male Nts:Mini rat, a transgenic rat carrying antisense RNA transgene for rat growth hormone

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Received 17 September 1998; received in revised form 22 February 1999; accepted 23 February 1999

Abstract

We investigated the characteristics of hepatic cytochrome P450s and flavin-containing monooxygenase 1 (FMO1) in male Nts:Mini rats, a Wistar/Jcl-derived transgenic rat strain showing less plasma GH concentration than the parental strain. The total hepatic P450 contents of Mini rats were significantly reduced. A suppression was observed in the activities and protein expression of male-specific P450s (CYP3A and CYP2C11) and was speculated to be a potential cause of the reduction in total P450 contents. The activity and protein expression of CYP2B1 were suppressed and those of CYP2E1 and CYP2B2 were enhanced. With the exception of our data on CYP2B1,these results largely agreed with previous reports concerning GH-depletion rat models (hypophysectomized rats, rats neonatally treated with glutamate, and dwarf rats), implying that the changes in Mini rats were caused by GH insufficiency. The liver FMO1 protein expression in Mini rats was higher than that in Wistar rats but the activity was comparable, suggesting that GH is not a positive regulator of FMO expression. With their insufficient but not depleted levels of plasma GH, Mini rats may thus become another candidate for use in the investigation of GH regulation of hepatic mixed-function monooxygenases. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cytochrome P450; Flavin-containing monooxygenase; Growth hormone deficiency; Liver microsome; Mini rats

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0378-4274/99/$ - see front matter © 1999 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0378-4274(99)00055-7

1. Introduction

Cytochrome P450 monooxygenases (P450s) are the major enzyme families for phase I metabolism of drugs and xenobiotics (Coon et al., 1996). Several P450 isozymes have been well known to be regulated by growth hormone (GH). To investigate GH regulation of P450 isozymes, previous researchers utilized several GH-depletion models including hypophysectomized rats (Hong et al., 1990), genetically GH-deficient dwarf rats (Shimada et al., 1995, 1997), and adult rats treated neonatally with monosodium glutamate (MSG) (Yamazoe et al., 1988; Pampori et al., 1991), an agent which allows for the nonsurgical suppression of adult GH levels. Through these types of investigation, it was found that male-specific isozymes of P450, such as CYP2C11 (Wells et al., 1994) and CYP3A (Oinonen and Lindros, 1995; Waxman et al., 1995), are down-regulated when there is a lack of pulsatile pattern of plasma GH concentration. The expressions of CYP2E1 and CYP2B1/2B2, on the other hand, are enhanced when GH is depleted (Yamazoe et al., 1989a,b).

Jcl:Wistar-TgN(ARGHGEN)1Nts rat (Uetsuka et al., 1997), or Nts:Mini rat (named Mini rat) (Matsumoto et al., 1995), is a recently developed transgenic rat in which the GH expression is suppressed by the presence of an antisense transgene for the rat GH gene located under the GH promoter gene expressed mainly in the pituitary (Matsumoto et al., 1993, 1995). The plasma GH level of Mini rats is reduced to approximately 60 and 80% in males and females, respectively, compared with those of sex- and age-matched Wistar/Jcl rats (Wistar rats), the parental strain of Mini rats (Matsumoto et al., 1995). As a result, Mini rats show retarded growth in comparison with Wistar rats (Tani et al., 1998) and are easy to handle. This rat strain is thought to have potential for a variety of uses, especially for studies concerning GH-related functions in vivo such as liver damage/regeneration (Uetsuka et al., 1997; Tani et al., 1998) or bone growth (Teranishi et al., 1998). However, constitutive expression and activities of P450 isozymes in Mini rats have not been reported. It is considered important to ascertain the ability to oxidize drugs and steroids in the application of this novel transgenic rat strain in a variety of animal experiments.

In addition to the P450 oxidizing system, an increasing number of reports have focused on the importance of flavin-containing monooxygenases (FMOs) in the oxidation of many xenobiotics containing nucleophilic nitrogen, sulfur, and phosphorous atoms (Ziegler, 1988). To date, at least five FMO forms have been reported in mammals (Lawton et al., 1994). Male rat liver contains FMO1 as a major form and FMO3 as a minor one (Moroni et al., 1995). FMOs are now thought to be involved in the metabolism of a variety of xenobiotics, as well as the P450 system (Damani and Nnane, 1996). However, very little has been learned about hormonal regulation of FMOs. Rat liver FMO protein appears to be positively regulated by testosterone and negatively controlled by estradiol (Dannan et al., 1986). Lemoine et al. (1991) reported that liver FMO protein expression was suppressed in hypophysectomized rats compared to untreated control rats, and that the activity was also reduced by hypophysectomy. In this context, it is of interest to know the FMO expression in the Mini rat, a strain which has inadequate amounts of plasma GH.

The aim of the present study was to determine the features of expression of several P450 isozymes and one of the FMO isozymes, FMO1, in the livers of male Mini rats, and to compare them with those in sex- and age-matched Wistar rats, the parental strain of Mini rats used as a control. In addition, we discussed whether the tendency of change in the expression of each of these monooxygenases in Mini rats was similar or dissimilar to the tendencies in other formerly investigated GH-deficient rat models such as hypophysectomized, dwarf, and MSG-treated rats.

2. Materials and methods

2.1. Animals

Male Wistar/Jcl (Wistar) rats (Clea Japan, Shizuoka, Japan) and Nts:Mini (Mini) rats (Nisseiken, Tokyo, Japan) were purchased at 4 weeks of age and acclimatized for at least 1 week before

the experiment. The animals were housed in stainless steel cages (four rats per cage) with sterile sawdust for bedding. The cages were kept in a specific pathogen-free facility with strict control of the temperature (23 ± 1°C), humidity (55 ± 5%), ventilation (10 times/h), and lighting period (07:00–19:00 h). The animals had free access to a standard commercial laboratory chow (F-2, Funabashi Farm, Chiba, Japan) and filtered tap water. All animals were cared for and treated humanely during the experiment in accordance with our laboratory animal care guidelines.

2.2. Chemicals and antibodies

7-Pentoxyresorufin, resorufin, β-NADPH, and glucose-6-phosphate were purchased from Sigma (St. Louis, MO). Erythromycin, aminopyrine, glucose-6-phosphate dehydrogenase, *p*-aminophenol and monomethylol dimethylhydantoin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Aniline was purchased from Ueno Chemical Industries (Osaka, Japan). Primary rabbit polyclonal antibodies against rat CYP2B1/2B2, CYP2E1, and CYP3A were included in respective Amersham's rat cytochrome P450 Western blotting kits (Amersham, Buckinghamshire, UK) together with secondary antibody, streptavidin–horseradish peroxidase conjugate, and enhanced chemiluminescence (ECL) detection reagent. Goat polyclonal antibody against rat CYP2C11 and secondary rabbit antibody against goat IgG were purchased from Daiichi Pure Chemicals (Tokyo, Japan). Polyclonal rabbit antibody to mouse liver FMO1 was prepared by A.K. All other materials used were of the highest quality commercially available.

2.3. Microsome preparation

The animals were killed at 6, 14, or 26 weeks of age by exsanguination under anesthesia with ether. Each group of both strains consisted of four rats. Microsomes were prepared in accordance with the method by Omura and Sato (1964) with minor modifications. Briefly, livers removed from rats were homogenized in 10 mM potassium phosphate buffer (KPB, pH 7.4) containing 1.15% potassium chloride (KCl). The homogenates were centrifuged at $9000 \times g$ and 4°C for 20 min, and the supernatant fluid was then ultracentrifuged at $105\,000 \times g$ and 4°C for 1 h. The re-suspended precipitates were ultracentrifuged again for 30 min, homogenized with KPB containing 30% glycerol, and stored at −80°C before use. Protein concentrations were determined according to the method reported by Lowry et al. (1951) using bovine serum albumin (Sigma) as the standard. Total contents of cytochrome P450 were photometrically measured according to the method of Omura and Sato (1964).

2.4. Enzyme activity assay

Aniline *p*-hydroxylase activity, mainly catalyzed by CYP2E1, was assayed by measuring the formation of *p*-aminophenol (Song et al., 1986). At final concentration, the incubation mixture (1 ml) contained 0.5 mg microsome, 0.1 mM EDTA, NADPH generating system (0.5 mM β-NADPH, 10 mM glucose-6-phosphate, 3 mM magnesium chloride, and 2 U/ml glucose-6-phosphate dehydrogenase), and 1 mM aniline as a substrate, in KPB (pH 7.4). *p*-Aminophenol was used as a standard. The mixture was incubated at 37°C for 20 min, and the reaction was stopped by adding cold trichloroacetic acid. After centrifugation, the supernatant was incubated with sodium carbonate and phenol at 37°C for 30 min, and the absorption at 630 nm was measured by a U-3300 spectrophotometer (Hitachi, Ibaraki, Japan).

Erythromycin and aminopyrine *N*-demethylase activities were assayed by measuring the formation of formaldehyde (Cooper and Brodie, 1955). At final concentration, the incubation mixture (1 ml) contained 1 mg microsome, 0.1 mM EDTA, and NADPH generating system, in KPB (pH 7.4). Monomethylol dimethylhydantoin was used as a standard. After pre-incubation at 37°C for 2 min, the reaction was initiated by the addition of 0.4 mM erythromycin or 1 mM aminopyrine, the mixture was incubated for another 20 min, and the reaction was stopped by adding cold trichloroacetic acid. After centrifugation, the supernatant was incubated with Nash's reagent (Nash, 1953) at 60°C for 30 min, and the absorption at 412 nm was measured by U-3300.

7-Pentoxyresorufin *O*-dealkylase activity, mainly catalyzed by CYP2B1/2B2 (Burke et al., 1985), was assayed by measuring the formation of resorufin (Clark et al., 1995). At final concentration, the incubation mixture (1 ml) contained 0.5 mg microsome and NADPH generating system in 20 mM Tris–HCl buffer (pH 7.8). Resorufin was used as a standard. After pre-incubation at 37°C for 2 min, the reaction was initiated by the addition of 20 μM 7-pentoxyresorufin, the mixture was incubated for another 5 min, and the reaction was stopped by adding cold methanol. After centrifugation, the supernatant was excited at 528 nm and the absorption of fluorescence at 590 nm was measured by an F-2000 fluorophotometer (Hitachi).

Benzydamine *N*-oxidase activity was assayed by measuring the formation of benzydamine *N*-oxide catalyzed mainly by FMO (Kawaji et al., 1993). The incubation mixture (0.3 ml) contained 0.5 mM NADPH and 0.17 mg microsome in 0.1 M Tricine–KOH buffer (pH 8.5). After incubation at 37°C for 5 min, the reaction was initiated by the addition of 0.25 mM benzydamine, the mixture was incubated for another 10 min, and the reaction was stopped by adding cold methanol. After centrifugation, the supernatant was subjected to high-performance liquid chromatography (HPLC) for determination of benzydamine and its metabolites according to the method reported by Baldock et al. (1990). The HPLC equipment was composed of a Shimadzu (Kyoto, Japan) LC-6A system with a fluorescence detector (Shimadzu RF-530, ex. 303 nm, em. 377nm) and a LiCrosorb RP-18 column (4 × 150 mm, Merck, Rahway, NJ). The mobile phase was methanol–acetonitrile–water–25% NH_4OH (50:40:10:0.05, v/v). The flow rate was 1.5 ml/min (0–3 min) and then 3 ml/min (3–20 min). Under the condition described above, benzydamine *N*-oxide and benzydamine were eluted with retention times of 2.1 and 5.1 min, respectively.

The activities of all enzymes except benzydamine *N*-oxidase were assayed in duplicate with individual microsomes. The benzydamine *N*-oxidase activity was assayed only once.

2.5. Western blot analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the method by Laemmli (1970) using 10% gels (TEFCO, Tokyo, Japan) for analyses of microsomal P450 isozymes and FMO1. Western blot analysis was carried out as described in previous reports (Towbin et al., 1979; Guengerich et al., 1982). Pooled microsomes (four rats/group) were examined in quadruplicate. The amounts of microsomes applied were 12.5 μg for CYP2B1/2B2, 10 μg for CYP2E1 and FMO1, 2.5 μg for CYP3A, and 0.5 μg for CYP2C11. Amersham's ECL cytochrome P450 Western blotting kit was utilized for the immunodetection, and the chemiluminescent signals were photographed with a Polaroid camera. Densitometry for quantification was performed using a Q550IW image analyzer (Leica, Tokyo, Japan). Quantified data were expressed relatively as percentages of 6-week-old Wistar rats.

2.6. Statistical analysis

Results are presented as the mean ± standard deviation (S.D.). Two-way analyses of variance with interaction (strain × age) and Fisher's protected least significant difference (LSD) test were used to determine the presence of significant differences between groups. All statistical analyses were performed using the SAS® system (SAS Institute, Cary, NC). The level of statistical significance employed in all cases was $P < 0.05$.

3. Results

3.1. Total cytochrome P450 content in the hepatic microsomes

The total P450 content in the hepatic microsomes was compared between Wistar and Mini rats at 6, 14 and 26 weeks of age (Fig. 1). The P450 total content was significantly lower in Mini rats than in Wistar rats, but the content was almost constant in both strains during this period.

3.2. Enzyme activities of hepatic cytochrome P450

The hepatic microsomal activities of 7-pentoxyresorufin *O*-dealkylase (Fig. 2A) decreased significantly in both strains at 14 and 26 weeks compared with 6 weeks, but the degrees of reduction were severer in Mini rats. The activities were comparable between the strains at 6 weeks of age, but they were significantly lower in Mini rats than in Wistar rats at 14 and 26 weeks.

The aniline *p*-hydroxylase activities were significantly higher in Mini rats than in Wistar rats, and in both strains the activities were significantly declined at 14 weeks and were restored at 26 weeks (Fig. 2B).

The erythromycin *N*-demethylase activities were comparable between the strains at 6 weeks of age, but they were significantly lower in Mini rats than in Wistar rats at 14 and 26 weeks (Fig. 2C). The activity in Wistar rats increased significantly at 26 weeks, while that in Mini rats significantly dropped at 14 weeks and thereafter.

The hepatic microsomal activities of aminopyrine *N*-demethylase were significantly higher in Wistar rats than in Mini rats throughout the period examined (Fig. 2D). The activities were practically constant in Wistar rats, but significantly diminished with age in Mini rats.

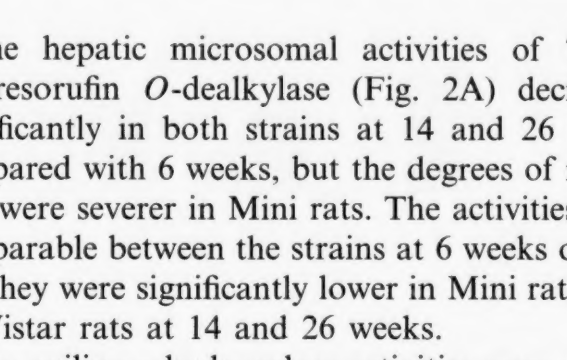

Fig. 1. Total hepatic cytochrome P450 contents in Wistar (■) and Mini rats (▩) at 6, 14 and 26 weeks of age. Data are represented as the mean ± S.D. of four rats. *Significantly different from age-matched Wistar rats ($P < 0.05$, Fisher's LSD test).

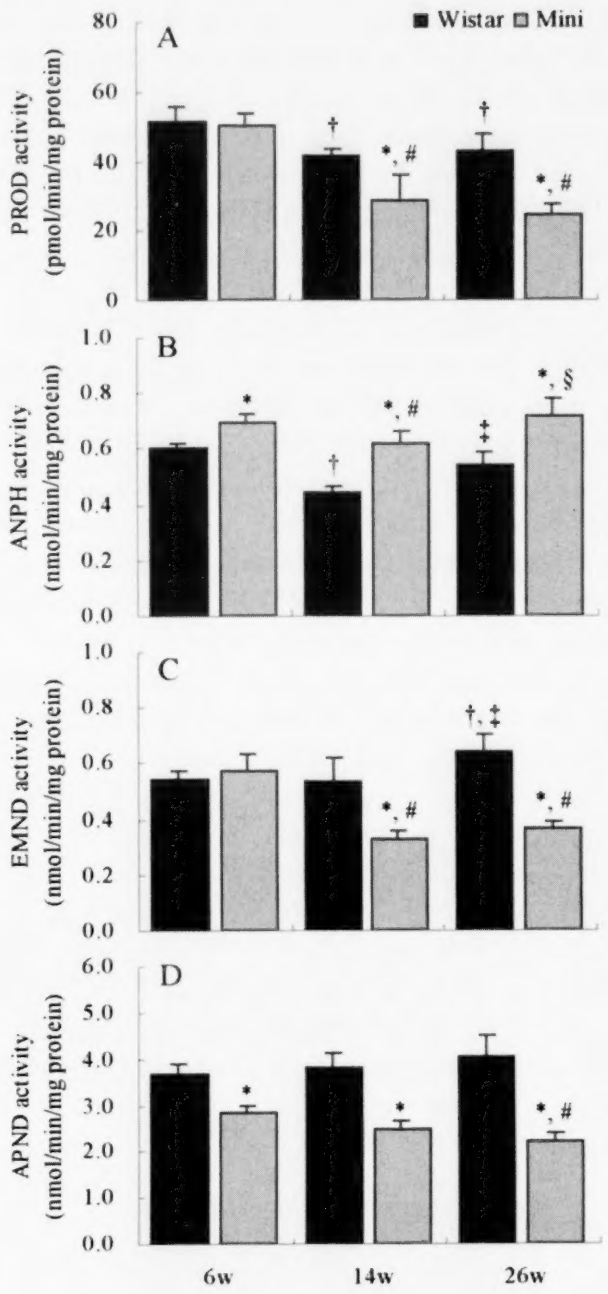

Fig. 2. Hepatic microsomal enzyme activities of 7-pentoxyresorufin *O*-dealkylase (A), aniline *p*-hydroxylase (B), erythromycin *N*-demethylase (C), and aminopyrine *N*-demethylase (D) in Wistar (■) and Mini rats (▩) at 6, 14 and 26 weeks. Data are represented as the mean ± S.D. of four rats. *Significantly different from age-matched Wistar, †from 6-week-old Wistar, ‡from 14-week-old Wistar, # from 6-week-old Mini, and §from 14-week-old Mini ($P < 0.05$, Fisher's LSD test).

3.3. Western blot analyses on hepatic cytochromes P450

Pooled hepatic microsomes of 6-, 14- and 26-week-old Wistar and Mini rats were probed with antibodies against rat CYP2B1/2B2, CYP2E1,

CYP3A and CYP2C11 (Fig. 3), and then visualized by the ECL system and quantified by an image analyzer (Fig. 4). Specific single bands corresponding to each P450 isozyme were observed in all age groups of both strains, with the exception of the anti-rat CYP2B1/2B2 (Fig. 3A), CYP2B1 protein band and higher (heavier) CYP2B2 protein band (Yamazoe et al., 1987).

The constitutive expression of CYP2B1/2B2 protein is low in rats, so at least 12.5 μg microsome per lane were required to visualize their immunoreactive bands (Fig. 3A). The CYP2B1 expression at 6 weeks was significantly higher than that at 14 and 26 weeks in Wistar rats, while the expression elevated with age in Mini rats (Fig. 4A). With these changes, CYP2B1 protein expressions in Mini rats were significantly lower than those in Wistar rats at 6 and 14 weeks, but at 26 weeks the immunoreactivities in both strains were comparable. The CYP2B2 expression was comparable in the two strains at 6 weeks, but dropped to significantly lower levels in Wistar rats at 14 and 26 weeks of age (Fig. 4B).

The expression of CYP2E1 protein (10 μg of microsomes were loaded) was significantly higher in Mini rats than Wistar rats from 6 to 26 weeks (Fig. 4C). In both strains the expression was significantly decreased at 14 weeks and restored at 26 weeks.

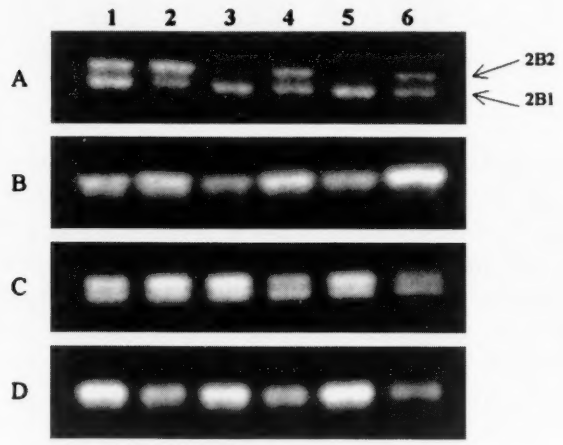

Fig. 3. Western blot analysis for CYP2B1/2 (12.5 μg/lane, A), CYP2E1 (10 μg/lane, B), CYP3A (2.5 μg/lane, C), and CYP2C11 (0.5 μg/lane, D) in hepatic microsomes isolated from Wistar and Mini rats. Lane 1, 6-week-old Wistar; lane 2, 6-week-old Mini; lane 3, 14-week-old Wistar; lane 4, 14-week-old Mini; lane 5, 26-week-old Wistar; and lane 6, 26-week-old Mini. Pooled microsomes from four rats per group were tested four times.

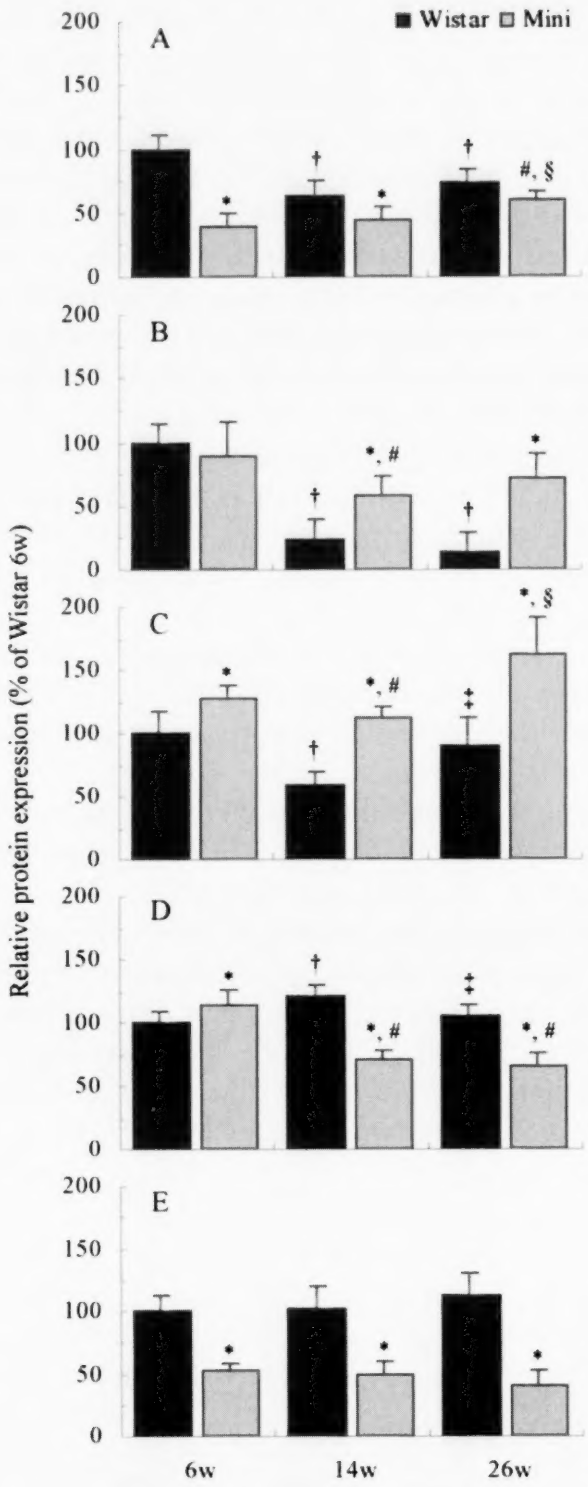

Fig. 4. Quantified data of Western blot analysis for CYP2B1 (A), CYP2B2 (B), CYP2E1 (C), CYP3A (D), and CYP2C11 (E) in hepatic microsomes isolated from Wistar (■) and Mini rats () at 6, 14 and 26 weeks. Data are represented as the mean ± S.D. of four analyses using pooled microsomes from four rats. For symbols for statistical significances, see Fig. 2.

Constitutive expressions of CYP3A and CYP2C11 are high in rats, and 2.5 and 0.5 μg of microsomes, respectively, were sufficient for anal-

ysis. In Wistar rats the CYP3A protein expression was higher at 14 weeks than at 6 and 26 weeks, while in Mini rats the expression was significantly decreased at 14 and 26 weeks (Fig. 4D). Although the expression was higher in Mini rats than in Wistar rats at 6 weeks, the results were reversed at 14 and 26 weeks (Fig. 4D). The CYP2C11 protein expression was significantly lower (approximately 36–50%) in Mini rats than in Wistar rats from 6 to 26 weeks, and the levels remained constant (Fig. 4E).

3.4. Hepatic FMO activity and Western blot analysis on hepatic FMO1

Hepatic microsomal FMO activity was measured as benzydamine *N*-oxidation, a reaction which is catalyzed by FMO but not by P450 (Kawaji et al., 1993). There were no statistically significant differences in the benzydamine *N*-oxidase activity of hepatic microsomes between Wistar and Mini rats at 6, 14 and 26 weeks of age (Fig. 5A).

Hepatic microsomes (10 μg) were probed with anti-mouse liver FMO1 and visualized by the ECL system (Fig. 5B). This antibody was cross-reacted with rat and mouse liver FMO, but not with recombinant human FMO3 (data not shown). A single band was observed in all age groups of both strains, and in each case its molecular weight was confirmed to be equal to that of the purified rat liver FMO used as a positive control (data not shown). When quantified, the FMO1 protein expressions in both strains, unlike benzydamine *N*-oxidase activity, showed significant decreases at 14 and 26 weeks compared with 6 weeks, and the expression in rats was significantly higher in Mini than in Wistar rats throughout the period examined (Fig. 5C).

4. Discussion

The present study disclosed the protein expressions and activities of several cytochrome P450s (CYP2B1/2B2, CYP2E1, CYP3A, and CYP2C11) and FMO1 in male Mini rats and compared them with those in Wistar rats.

The total content of hepatic P450 in Mini rats was significantly lower than that in non-transgenic Wistar rats at 6, 14 and 26 weeks of age. In addition, Western blot analysis revealed dramatic decreases in the expression of hepatic CYP2C11 protein in Mini rats compared with that in Wistar rats at all ages examined. Decreases in total P450 contents have been shown in other GH-depletion models such as dwarf (Shimada et al., 1997), hypophysectomized, and MSG-treated rats (Pampori and Shapiro, 1994), and in male rats, these decreases are associated with decreases in CYP2C11, a male-specific P450 isozyme which occupies approximately 50% of the total P450 in males (Morgan et al., 1985). These previous reports strongly support our data, and the reductions in the hepatic contents of total P450 and

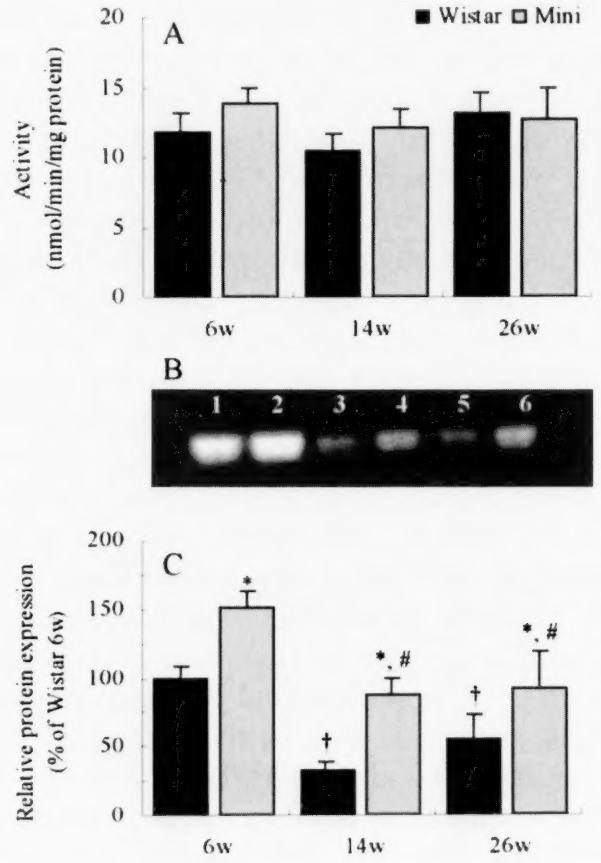

Fig. 5. Hepatic microsomal benzydamine *N*-oxidation activity (A), Western blot analysis for FMO1 (10 μg/lane, B), and quantified data of Western blot analysis for FMO1 (C) of Wistar (■) and Mini rats (▩) at 6, 14 and 26 weeks. Data are represented as the mean ± S.D. of four rats in (A) or four analyses using pooled microsomes from four rats in (C). For symbols of statistical significances in (A) and (C), see Fig. 2. Lane numbers in (B) correspond to those in Fig. 3.

CYP2C11 protein in male Mini rats appear to be caused by the suppression of plasma GH levels resulting from the transgene expression.

Aminopyrine is known to be metabolized by multiple forms of cytochrome P450 into *N*-demethylated and/or 3-hydroxylated metabolites. Imaoka et al. (1988) reported that CYP2C11 and CYP2B1 are mainly responsible for *N*-demethylation of aminopyrine. It has been reported that the expression of the constitutive CYP2B1 protein is very low (Yamazoe et al., 1987, 1989a). In this study, 25 times more microsomes were loaded for CYP2B1 than for CYP2C11, but the Western blot analysis still showed that the immunoreactivities of the CYP2B1 protein were relatively weaker than those of CYP2C11, suggesting that the CYP2B1 was present in far lower quantities than CYP2C11. Given these findings, CYP2C11 can be considered the main catalyzer in aminopyrine *N*-demethylation activity among the many forms participating in the reaction. The present data indicate that aminopyrine *N*-demethylation activity was significantly suppressed in Mini rats, supporting the reduction of CYP2C11 protein in Mini rats shown by Western blot analysis.

CYP3A is another male-specific P450 isozyme and composes approximately one-fourth of the total hepatic microsomal P450 in Wistar rats (Imaoka et al., 1991). In rats with the complete depletion of serum GH caused by neonatal MSG treatment, CYP3A protein and activity were suppressed (Pampori et al., 1991; Waxman et al., 1995), and pulsatile GH administrations restored the suppressed CYP3A levels (Waxman et al., 1995). Shimada et al. (1997) also reported that Sprague–Dawley-derived dwarf rats show lower levels of CYP3A protein and testosterone 6β-hydroxylase activity compared to the normal strain. On the other hand, it has been reported that CYP3A expression is induced in hypophysectomized rats (Oinonen and Lindros, 1995; Waxman et al., 1995). In this study, results of Western blot analysis for CYP3A well corresponded to the erythromycin *N*-demethylase activity, a marker of CYP3A. Although there were no or little differences in enzyme activity or protein expression between Wistar and Mini rats at 6 weeks, both of these parameters were suppressed in Mini rats at 14 and 26 weeks. Therefore, in respect to CYP3A expression, Mini rats seem to be comparable to dwarf and MSG-treated rats, but not to hypophysectomized rats. In addition, the reduction in CYP3A expression in Mini rats is also thought to effect the reduction in the total P450 contents in this strain.

CYP2E1 is known as an ethanol-inducible P450 monooxygenase (Song et al., 1986), and it is thought to be responsible for a number of toxicologically important reactions, such as the metabolic activations of nitrosoamines (Patten et al., 1986), acetaminophen (Sato et al., 1981), and carbon tetrachloride (Watkins et al., 1988). It has been reported that the expression of CYP2E1 is up-regulated both in hypophysectomized rats (Yamazoe et al., 1989b; Hong et al., 1990) and in dwarf rats (Shimada et al., 1995). The data obtained in the present study well agreed with the previous results. Both the enzyme activation and protein expression of Mini rats were higher than those of Wistar rats in all ages examined. Taken together, plasma GH is thought to be a suppressive factor for the CYP2E1 expression.

CYP2B1 and 2B2 are noted as phenobarbital-inducible cytochrome P450 isozymes (Waxman and Walsh, 1982). The expressions of CYP2B1and CYP2B2 protein have been reported to be induced at much higher rates in hypophysectomized (Yamazoe et al., 1987, 1989a; Murayama et al., 1991) and dwarf (Shimada et al., 1997) rats than in respective normal strains. GH is believed to act as a repressive factor for the enzyme (Yamazoe et al., 1987). However, it has been described, on the contrary, that 7-pentoxyresorufin *O*-dealkylase activity, a marker enzyme of CYP2B1/2B2 (Burke et al., 1985), is reduced in MSG-treated (Yamazoe et al., 1988) rats and hypophysectomized (Yamazoe et al., 1987) rats. In the Mini rats in the present study, the activity of the enzyme and the expression of the CYP2B1 protein were suppressed, and the expression of the CYP2B2 protein was up-regulated. The mechanisms which lowered the CYP2B1 protein expression in Mini rats are not known. It has been assumed that CYP2B proteins are regulated not only by GH, but also by thyroid hormone (Murayama et al., 1991). Moreover, the kinetic

parameters of hormones other than GH in Mini rats have not been yet investigated. Further studies will be needed to clarify the mechanism(s) of regulation of CYP2B1 in Mini rats.

The flavin-containing monooxygenase (FMO) is another phase I drug-metabolizing system in the liver. In male rat liver, at least two isozymes are known to be expressed: FMO1, a major form, and FMO3, a minor one (Moroni et al., 1995). However, little is known about the regulation of FMO expression in rat liver by pituitary and gonadal hormones. In a previous study showing that hepatic FMO is suppressed by hypophysectomy, the masculine type of GH supplement could not restore the reduced levels of FMO expression, but testosterone administration partially reversed the FMO activity (Lemoine et al., 1991). It has been also reported that the FMO expression is suppressed and enhanced in birth-gonadectomized males and females, respectively, and that testosterone supplement up-regulates the FMO expression both in gonadectomized males and females (Dannan et al., 1986). Taken together, it has been hypothesized that testosterone, not GH, is the positive regulator of the FMO expression.

The benzydamine *N*-oxidation activity reflects mainly the activity of FMO but not P450 (Kawaji et al., 1993). Although this activity is not isozyme-specific for FMO, it may reflect the activity of FMO1, since FMO3 is a minor form in rat liver. By showing that the FMO activity in Mini rats did not differ significantly from that in Wistar rats and that the protein expression in Mini rats was up-regulated compared with that in Wistar rats, the present study supports the hypothesis that GH is not likely to play an important role in the FMO regulation in this strain. Further studies on gonadal hormones in Mini rats are anticipated.

This study provides some basic information on hepatic cytochrome P450 isozymes (CYP2B1/2B2, CYP2E1, CYP3A and CYP2C11) and FMO in Mini rats, a novel transgenic rat strain with suppressed levels of plasma GH. The results obtained here, together with the regulation of P450s in the other GH-depleted models reported previously, suggest that, compared with the three previously developed models, the characteristics of P450s expression in Mini rats are concordant in some isozymes and different in others. Therefore, Mini rats are thought to comprise another GH-deficient rat model, in addition to hypophysectomized, dwarf, and MSG-treated rats, for use as a new tool in the investigation of GH regulation of hepatic P450 expression.

Acknowledgements

The authors wish to thank Simon A. Johnson for reading the manuscript, and Yasushi Orihashi for his professional advice on statistics.

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Toxicology Letters 106 (1999) 171–180

Toxicology Letters

Evaluation of rat hepatic 2E1 activity in function of age, sex and inducers: choice of an experimental model capable of testing the hepatotoxicity of low molecular weight compounds

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Received 25 November 1998; received in revised form 22 February 1999; accepted 26 February 1999

Abstract

The aim of this work on rat hepatic P450 2E1 activity was to seek the most suitable experimental model to study the role of cytochrome P450 2E1 in the metabolism of industrial chemicals. Two sets of experiments were devoted to selecting the age and sex of animals and to estimating the response of male and female rats to different inducers. In the first set, the effect of three inducers (fasting; ethanol; acetone) was studied in male rats aged 5, 7 and 9 weeks. In the second set, the effect of different inducers, namely β-naphthoflavone (BNF), phenobarbital (PB), ethanol, acetone and pyridine, on PNP and chlorzoxazone (CLZO) hydroxylase activities was studied in 7 week old male and female rats. The results demonstrate firstly that microsomal *p*-nitrophenol (PNP) hydroxylase activity significantly decreases in control male rats in inverse function of age, and secondly that induction by ethanol decreases with age. The PNP hydroxylase activity level of controls and the significant increases in PNP hydroxylase activity observed in 7 week old male rats show that this is the most suitable age for the second set of experiments. In this second set, it was shown that P450 1A (induced by BNF) is involved in CLZO hydroxylase activity only. PB increased the hydroxylase activities in male and female rats by about 1.5 and 1.7 times those of the controls, respectively. The effects of P450 2E1 inducers in function of sex show that male rats exhibited more significant increases in PNP and CLZO hydroxylase activities than female. The specificity of these two substrates is discussed. Neither of these two reactions was specifically catalysed by P450 2E1, but PNP may be considered as the most specific and the least sensitive substrate. In addition, the linear relationship observed between the two substrates (PNP and CLZO) showed a good correlation between their activities ($r = 0.90$, $P < 0.001$). In conclusion, these results suggest the use of the 7 week old male rat as the experimental model to study the role of cytochrome P450 2E1 in the hepatotoxicity of low molecular weight industrial chemicals. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Activity; Cytochrome P450 2E1; Liver; Rat; Inducers

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PII: S0378-4274(99)00058-2

1. Introduction

Cytochromes P450 are a superfamily of hemoproteins that are involved in the oxidative metabolism of a large number of endogenous and exogenous compounds (Wrighton and Stevens, 1992). The liver microsomal P450 enzymes involved in xenobiotic biotransformation belong to three main P450 families (P450 1, 2, 3), (Parkinson, 1996). Among these, the 2E subfamily is considered as a very important metabolic system that catalyses the bioactivation of numerous industrial chemicals in the low molecular weight range (benzene, styrene, trichlorethylene etc.) (Wrighton and Stevens, 1992; Gonzalez and Gelboin, 1994). In humans and in rats, the 2E subfamily is only represented by the 2E1 isoenzyme (Wrighton and Stevens, 1992). Immunoinhibition, immunoquantitation and structural studies have demonstrated that the liver P450 2E1 from rat and human are highly similar (Wrighton et al., 1986; Wrighton and Stevens, 1992). Thus, the rat appears to be an excellent model to study human P450 2E1 expression and function (Wrighton and Stevens, 1992). This P450 2E1 is found principally in the liver, but also exists in several extrahepatic tissues (kidney, lung etc.), (Raucy et al., 1993; Nedelcheva and Gut, 1994).

The regulation of P450 2E1 enzyme levels involves both endogenous and exogenous factors (Raucy et al., 1993). Endogenous regulation of 2E1 entails physiological conditions such as fasting (Tu and Yang, 1983; Ueng et al., 1993) and diabetes (Peng et al., 1983). Exogenous factors include exposure to xenobiotics such as ethanol, acetone (Peng et al., 1982; Yoo et al., 1987) and drugs which can result in a marked induction of P450 2E1 enzyme levels (Raucy et al., 1993). Several mechanisms have been proposed to understand its regulation which includes increases in 2E1 mRNA stemming from transcriptional activation or mRNA stabilization, an increase in mRNA translatability, and a decrease in protein degradation (Koop and Tierney, 1990; Parkinson, 1996). The principal mechanism that controls the induction process depends on the chemical nature of the inducers, the age and the nutritional and hormonal status of the animal (Koop and Tierney, 1990). Acetone and ethanol induce P450 2E1 activity by decreasing protein degradation (Johansson et al., 1988; Song et al., 1989; Koop and Tierney, 1990; Ronis et al., 1991). On the other hand, starvation (Johansson et al., 1988; Koop and Tierney, 1990; Hu et al., 1995) or diabetis (Johansson et al., 1988; Song et al., 1989; Koop and Tierney, 1990; Ronis et al., 1991) induce P450 2E1 activity by means of two stabilization mechanisms of mRNA and P450 2E1 protein.

Cytochrome P450 2E1 activity can easily be determined with a few sensitive and specific substrates. Among these, 4-nitrophenol (PNP) (Koop, 1986), and chlorzoxazone (CLZO) (Peter et al., 1990; Carriere et al., 1993) are widely used. Hydroxylation of PNP to 4-nitrocatechol and of CLZO to 6-hydroxychlorzoxazone by the P450 2E1 enzyme is a well known specific reaction, and certain endogenous and exogenous factors have been shown to increase its activity. However, the numerous results described in the literature are partial and dissimilar (Lorr et al., 1984; Johansson et al., 1988; Brown et al., 1995; Dekant et al., 1995; Hu et al., 1995; Paolini et al., 1996). The aim of this work on rat hepatic P450 2E1 activity was therefore to seek the most suitable experimental parameters to study the role of cytochrome P450 2E1 in the hepatotoxicity of low molecular weight industrial chemicals. To achieve this, two experiments were conducted to select the age and sex of animals, to estimate the response of different inducers, and to compare the results and the specificity of these two substrates.

2. Material and methods

2.1. Chemicals

All chemicals were of the highest available quality. These comprised CLZO, PNP, 4-nitrocatechol, β-naphthoflavone (BNF) and DL-isocitric acid trisodium salt which were purchased from Sigma Chemical Co (St. Louis, MO). Sodium phenobarbital (PB) was obtained from Fluka Chemika (Switzerland) and NADPH, $NADP^+$ and isocitric dehydrogenase were obtained from Boehringer (Mannheim). Solvents from Merck

(Darmstadt) were of HPLC grade. The 6-hydroxy-CLZO and 5-fluoro-benzoxazolone were synthesized by an original method developed in our laboratory (Cossec et al., 1999), and the purity of each compound was 99.5 and 99.7%, respectively.

2.2. Experimental design

Male and female Sprague–Dawley rats (Iffa-Credo, France) were acclimatized for at least 5 days at 22°C with a 12-h dark–light cycle, and had free access to food and water. In the first experiment, three groups of twenty male rats aged 5, 7 and 9 weeks with a mean weight of 120, 190 and 290 g, respectively were used. Each group was composed of four subgroups; a control group, a group that fasted for one day, an acetone group (5 ml/kg of 25% solution administered by single gavage 24 h before sacrifice) (Lorr et al., 1984), and an ethanol group (15% in drinking water for 6 days) (Peng et al., 1982).

In the second experiment, two groups of 7 week old rats, each comprising sixty male and female animals (weighing about 200 and 160 g, respectively), were used for the treatments. In each group, 12 subgroups of five animals were used for six treatments (inducers) and their respective controls. BNF (80 mg/kg per day) was given i.p. for 4 days in corn oil (4 ml/kg) (Moorthy et al., 1995), and PB (80 mg/kg per day) was given i.p. for 4 days in saline solution (2 ml/kg) (Gut et al., 1993). Ethanol (15% in the drinking water) was given for 6 days (Peng et al., 1982). Acetone (25% in water) was administered by single gavage (5 ml/kg) (Lorr et al., 1984). The fasting treatment lasted one day. Pyridine (200 mg/kg per day) was given i.p. in saline solution (2 ml/kg) for 4 days (Chaney and Carlson, 1995). For each treatment, a control subgroup received the same vehicle in the same conditions as the corresponding treated subgroup. The animals were anesthetized by a single injection of pentobarbital (60 mg/kg) and exsanguined by abdominal aorta section. The livers were excised, rinsed and immediately stored in liquid nitrogen until microsomal preparation.

2.3. Microsomal preparation

The livers were defrosted and homogenized at 4°C with 0.05 M sodium phosphate buffer pH 7.4 containing 0.25 M sucrose, 10 mM EDTA and 1 mM dithiothreitol (DTT). The homogenates were centrifuged at $1000 \times g$ for 10 min then at $7000 \times g$ for 20 min, and the supernatant was again centrifuged at $105\,000 \times g$ for 1 h. The pellet was resuspended and washed with 0.1 M tetrasodium pyrophosphate buffer pH 7.4 containing 10 mM EDTA and 0.1 mM DTT, and centrifuged at $105\,000 \times g$ for 1 h. The microsomes were resuspended in 0.1 M potassium phosphate buffer pH 7.4 containing 1 mM EDTA, 1 mM DTT and 20% glycerol to a final concentration of about 15 mg of protein/ml. The microsomes were stored in small aliquots at -20°C until analysis. Microsomal protein concentration was determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard.

2.4. PNP hydroxylase activity

The hydroxylation of PNP to 4-nitrocatechol was carried out as described by Koop (1986). A 0.2 mM substrate concentration was used, and the 4-nitrocatechol formed was determined by absorbance at 546 nm. A NADPH-generating system consisting of $NADP^+$, sodium isocitrate and isocitrate dehydrogenase was used (Reinke and Moyer, 1985).

2.5. CLZO hydroxylase activity

The hydroxylation of CLZO to 6-hydroxy-CLZO was carried out as described by Peter et al. (1990). A 0.4 mM substrate concentration was used, and the 6-hydroxy-CLZO formed was measured by an HPLC method with 5-fluoro-benzoxazolone as internal standard. NADPH 1 mM served as a substitute for the NADPH-generating system (Carriere et al., 1993).

2.6. Statistics

The results are expressed as mean $\pm$ S.E., and the U of Mann–Whitney test was employed to

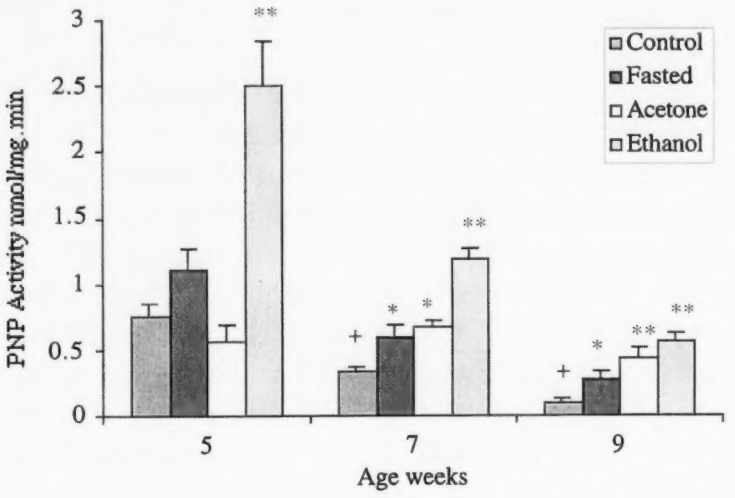

Fig. 1. Effect of the age of male rats on microsomal *p*-nitrophenol hydroxylase activity induced by one day of fasting, acetone (5ml/kg of 25% solution) injected by single gavage (24 h before sacrifice), and ethanol (15% in drinking water) for 6 days. Data represent means ± S.E. of five rats per group. The comparison between control and treated groups was made using one way analysis of variance, followed by Student–Neuwman–Keuls test. Significantly different from control group: $*P = 0.05$; $**P = 0.01$. The comparison between the three control groups was made using the same test. Significantly different from control group aged 5 weeks: $+P < 0.01$.

compare a single treatment and its corresponding control. The comparison between control and treated groups was made using one-way analysis of variance followed by Student Neuwman–Keuls test, and the level of significance was $P = 0.05$ and 0.01.

3. Results

The data on the effect of the age of male rats on the microsomal PNP hydroxylase activity induced by fasting, acetone and ethanol are shown in Fig. 1. PNP hydroxylase activity significantly decreased in the control male rats in inverse function of age. The increase in PNP hydroxylase activity observed in fasted rats was significant, except in the 5 week old rats. Acetone treatment induced PNP hydroxylase activity significantly only in rats aged 7 and 9 weeks. Among the three inducers used, ethanol was the strongest whatever the age of the animals, and the increase in activity was always significant, but decreased with the age of the animal. The activity level of the control rats and the significant increases in PNP hydroxylase activity observed with the three treatments would suggest that 7 week old animals should be used for the second experiment.

The effects of various inducers on microsomal PNP and CLZO hydroxylase activities in the male rat are shown in Table 1. In control male rats the ranges the two hydroxylase activities were from 0.41 to 0.46 and from 0.47 to 0.87 nmol/mg protein min with PNP and CLZO as substrate, respectively. In male rats treated with BNF, only the CLZO hydroxylase activity increased significantly by a factor of 4.7. PB treatment significantly increased the hydroxylase activities

Table 1
Effect of various inducers on microsomal *p*-nitrophenol and chlorzoxazone hydroxylase activities in the male rat[a]

Substrate	*p*-Nitrophenol		Chlorzoxazone	
Group	Treated	Control	Treated	Control
β-naphthoflavone	0.37 ± 0.023	0.41 ± 0.019	3.04 ± 0.13**	0.64 ± 0.038
Phenobarbital	0.75 ± 0.038**	0.46 ± 0.034	0.98 ± 0.079**	0.52 ± 0.066
Day of fasting	0.84 ± 0.093*	0.42 ± 0.071	1.02 ± 0.15	0.87 ± 0.090
Acetone	1.15 ± 0.085*	0.42 ± 0.030	1.11 ± 0.033* (4)	0.77 ± 0.042
Ethanol	1.81 ± 0.16**	0.42 ± 0.071	1.49 ± 0.079**	0.87 ± 0.090
Pyridine	2.07 ± 0.27*	0.43 ± 0.017	2.34 ± 0.13**	0.47 ± 0.017

[a] Activity is expressed in nmol/mg protein min. Data represent means ± S.E. of five rats per group, except when the number is in brackets. The comparison between control and treated groups was made using U of Mann–Whitney test. Significantly different from corresponding control group.

* $P < 0.05$.

** $P = 0.01$.

Table 2
Effect of various inducers on microsomal *p*-nitrophenol and chlorzoxazone hydroxylase activities in the female rat[a]

Substrate	*p*-Nitrophenol		Chlorzoxazone	
Group	Treated	Control	Treated	Control
β-naphthoflavone	0.20 ± 0.028**	0.54 ± 0.085	3.18 ± 0.14**	0.67 ± 0.093
Phenobarbital	0.99 ± 0.17	0.65 ± 0.12	0.74 ± 0.10*	0.46 ± 0.037
Day of fasting	0.56 ± 0.084	0.49 ± 0.02	0.60 ± 0.036**	0.39 ± 0.017
Acetone	0.47 ± 0.11	0.45 ± 0.068	0.54 ± 0.025* (4)	0.38 ± 0.032
Ethanol	0.94 ± 0.12	0.49 ± 0.02	1.03 ± 0.081**	0.39 ± 0.017
Pyridine	2.62 ± 0.29**	0.65 ± 0.12	2.66 ± 0.15**	0.46 ± 0.037

[a] Activity is expressed in nmol /mg protein min. Data represent means ± S.E. of five rats per group, except when the number is in brackets. The comparison between control and treated groups was made using U of Mann–Whitney test. Significantly different from corresponding control group.

* $P = 0.05$.

** $P = 0.01$.

evaluated with both substrates. These activities were 1.6 and 1.9 times those of the controls with PNP and CLZO, respectively. Treatment with the four inducers (fasting, acetone, ethanol and pyridine) increased both microsomal hydroxylase activities significantly except for fasting with CLZO as substrate. The PNP hydroxylase activities observed with fasting, acetone, ethanol and pyridine were 2, 2.7, 4.3 and 4.8 times higher than the corresponding controls, and the CLZO hydroxylase activities were 1.2, 1.4, 1.7 and 4.9 times those of the controls.

The data observed with the same treatment in female rats are shown in Table 2. In control female rats the ranges the two hydroxylase activities were from 0.45 to 0.65 and from 0.38 to 0.67 nmol/mg protein min with PNP and CLZO as substrate, respectively. As for the males, BNF significantly increased CLZO hydroxylase activity by the same factor (4.7). In the females, PNP hydroxylase activity decreased significantly whereas in the males this activity was not modified. In female rats treated with PB, only CLZO hydroxylase activity increased significantly, and the increase in PNP hydroxylase activity was not significant because of the large dispersion of results. These activities were 1.3–1.8 times and 1.6 times those of the controls with PNP and CLZO, respectively. In female rats treated with the four inducers of P450 2E1 (fasting, acetone, ethanol, pyridine), CLZO hydroxylase activity increased significantly, and only pyridine treatment increased PNP hydroxylase activity significantly. In contrast, in the males the two activities increased significantly with the four inducers except for fasting with CLZO as substrate. The increases in activity observed with ethanol treatment in female rats were 1.9 and 2.6 times those of the controls with PNP and CLZO, respectively. Those observed with pyridine treatment were four times and 5.8 times those of the controls with PNP and CLZO, respectively. CLZO hydroxylase activity was 1.4 and 1.5 times higher than the controls with acetone treatment and fasting, respectively. In the males, the inductions observed with the three inducers (fasting, acetone and ethanol) were more significant than those observed in the females.

The results of Tables 1 and 2 demonstrate that P450 1A (induced by BNF) is involved in CLZO hydroxylase activity. On the other hand, PB increases the hydroxylase activities significantly in both male and female rats, except in females with PNP as substrate where the increase is not significant because of the large dispersion of results. The effects of P450 2E1 inducers in function of sex show that the males exhibit more significant increases in PNP and CLZO hydroxylase activities than the females. The correlation coefficient obtained for the comparison of CLZO 6-hydroxyla-

tion and PNP hydroxylation was found to be highly significant ($P = 0.001$, $r = 0.90$; Fig. 3). All the individual values of Tables 1 and 2, except the values of BNF treated animals, were used for this correlation. The line of best fit was determined by linear regression analysis, with a slope of 0.959 and an intercept of -0.029 which is not significant ($P = 0.60$). There is a good linear correlation between the results observed with the two substrates (PNP and CLZO), and this correlation is not sex-dependent.

Seven substrate concentrations ranging from 40 to 800 μM were used to determine the kinetics parameters (K_m and V_m) of the CLZO hydroxylase activity in the control rats (Fig. 2). The curve was found to follow a biphasic Michaelis–Menten kinetic, characterized by a low K_m of 75 μM and V_m of 1.10 nmol/mg protein min for the 40 to 240 μM substrate range, and a high K_m of 253 μM and V_m of 1.64 nmol/mg protein min for the 240 to 800 μM substrate range.

4. Discussion

The P450 2E1 activity modifications related to age, sex and administration of certain inducers have never been studied simultaneously. A number of studies on rats have demonstrated that several inducers appear to increase hepatic cytochrome P 450 2E1 activity (Peng et al., 1982; Yoo et al., 1987; Chaney and Carlson, 1995), and that this activity depends on age (Thomas et al., 1987; Manjgaladze et al., 1993). Moreover, it has been demonstrated that several cytochrome P450 could be involved in CLZO and PNP hydroxylation (Carriere et al., 1993; Goasduff et al., 1996; Jayyosi et al., 1995; Ono et al., 1995; Yamazaki et al., 1995; Gorski et al., 1997; Zerilli et al., 1998). However, there are only two reports on male rats that partially compare the results obtained with the two substrates (CLZO and PNP) in control or induced rats by pyrazole, pyridine, acetone and 3-methylcholantrene (Goasduff et al., 1996), and by pyrazole and dexamethazone (Zerilli et al., 1998).

The results of this study on Sprague–Dawley rats indicate that the PNP hydroxylase activity of hepatic microsomal fraction significantly diminishes by a factor of 7 between control rats aged from 5 to 9 weeks. This confirms the earlier findings of Manjgaladze et al. (1993), who observed a two-fold decrease in Fisher (F344) male rats between 18 weeks and 22 months of age, and those of Thomas et al. (1987), who indicated what the P450 2E1 content decreases between 3 and 6

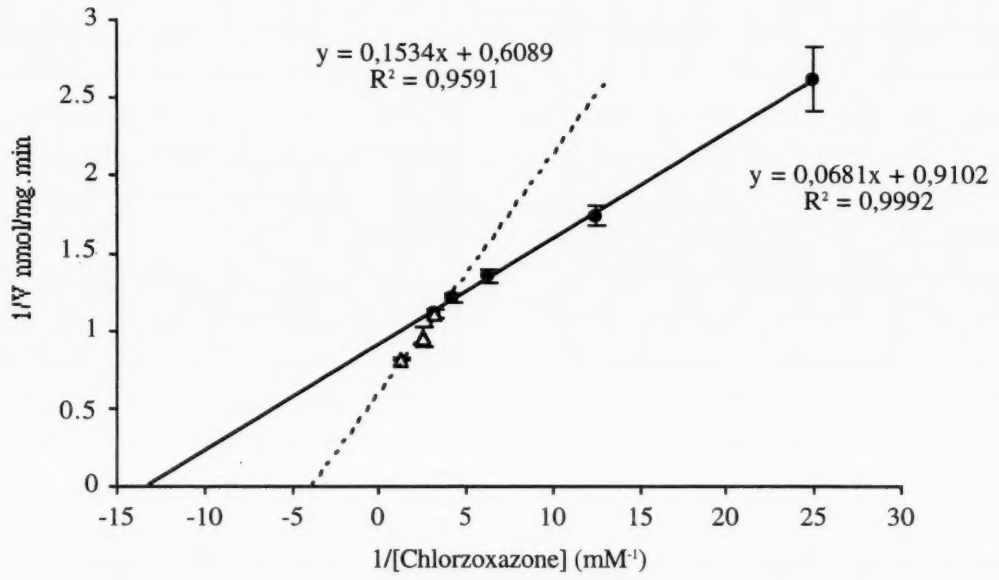

Fig. 2. Double reciprocal plots. Chlorzoxazone hydroxylase activity was assayed using microsomes from control rat liver (0.4 mg/ml incubation). A chlorzoxazone concentration range from 0.04 to 0.8 mM was used. Points were means and S.D. of three determinations. Kinetic parameters were determined by linear regression analysis: ● $K_m = 75$ μM and $V_m = 1.10$ nmol/mg min; $\Delta K_m = 253$ μM and $V_m = 1.64$ nmol/mg min.

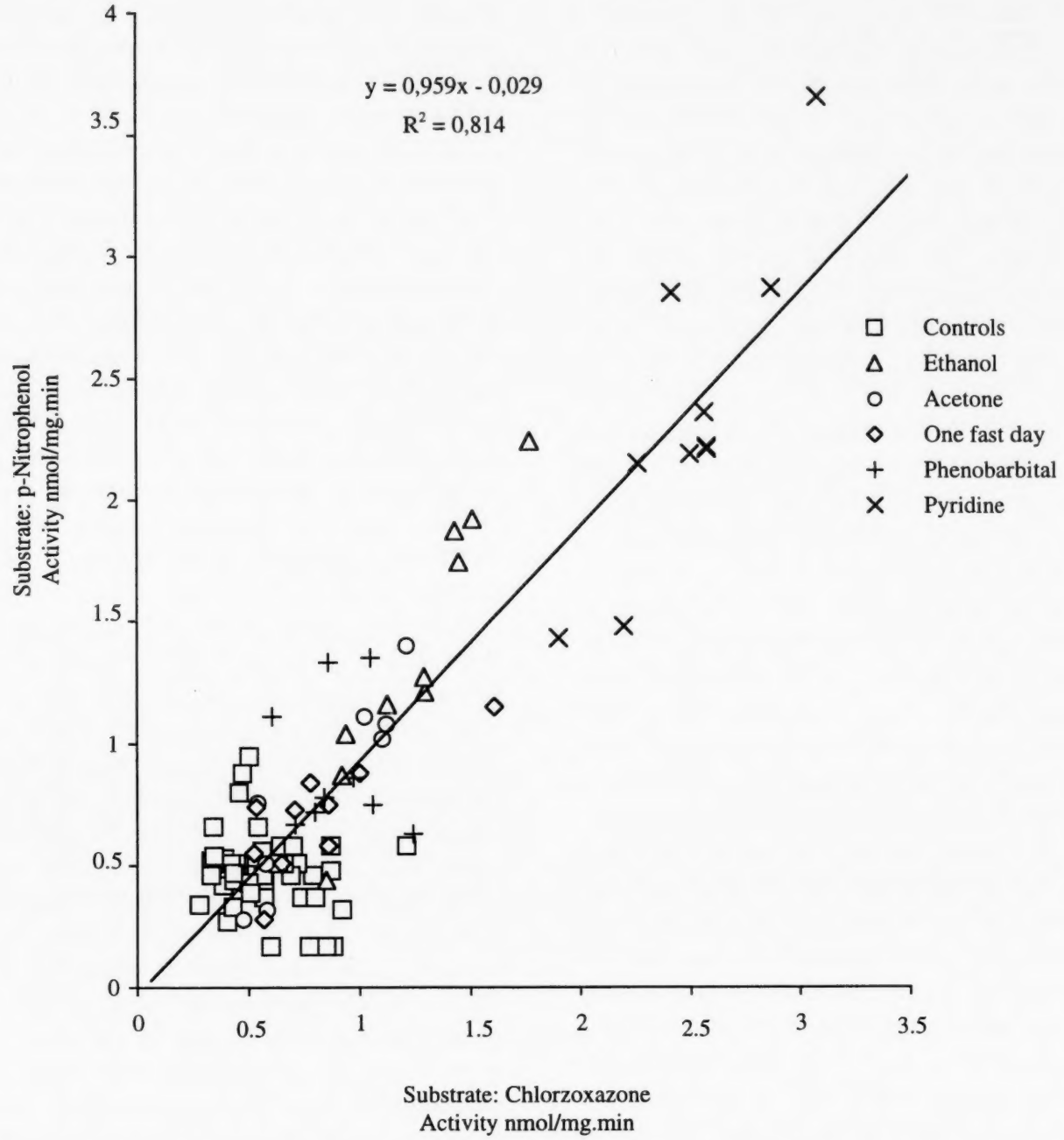

Fig. 3. Correlation analysis between microsomal hydroxylase activities for *p*-nitrophenol and chlorzoxazone as substrates. Microsomes from control, fasted, acetone, ethanol, phenobarbital and pyridine male and female rats were used ($n = 92$).

weeks of age. Whatever the age of the animals, PNP hydroxylase activity increased after one day of fasting, which is consistent with the results of Brown et al. (1995) observed in male F344 rats. Of three inducers tested, ethanol is the strongest whatever the age of the animals, and PNP hydroxylase activity increases by an identical factor of about 3.5 for animals aged 5 and 7 weeks.

In control rats, the PNP and CLZO hydroxylase activities are probably not sex-dependent, but there are some discrepancies in the literature (Dekant et al., 1995; Jayyosi et al., 1995; Paolini et al., 1996). The PNP hydroxylase activity was 0.43 ± 0.017 (mean $\pm$ S.E.) and 0.53 ± 0.040 nmol/mg protein min, respectively for male and female control rats, and the CLZO hydroxylase activity was 0.66 ± 0.038 and 0.48 ± 0.036. The high degree of variability observed with CLZO as substrate (Tables 1 and 2) in the control rats may be explained by a number of factors:

(a) the inter-individual variability; (b) the fact that in our experimental conditions some cy-

tochromes P 450 (2E1, 1A and 3A) were involved in CLZO hydroxylation; (c) the effect of the different vehicles used. Our values are in agreement with the activities observed in the literature (in nmol/mg protein min; mean ± S.D.): for male rats, these values are 1.15 ± 0.11 (Paolini et al., 1996) for PNP and 0.41 ± 0.019 (Dekant et al., 1995) and 2.42 ± 1.02 (Jayyosi et al., 1995) for CLZO; for female rats, 0.85 ± 0.06 (Paolini et al., 1996) and 0.43 ± 0.028 (Dekant et al., 1995) for PNP and 1.60 ± 0.15 (Jayyosi et al., 1995) for CLZO.

In both males and females, BNF treatment increased the CLZO hydroxylase activity, but the PNP hydroxylase activity did not increase. BNF preferentially induced P450 1A1(Guengerich et al., 1982), which is involved in CLZO 6-hydroxylation activity (Carriere et al., 1993; Yamazaki et al., 1995), but not in PNP hydroxylation activity. This confirms the earlier results observed with 3-methylcholanthrene as inducer of P450 1A1(Goasduff et al., 1996). In the treated rats, BNF stimulated CLZO 6-hydroxylation to a greater degree.

In the control rats, the kinetic parameter study showed that CLZO 6-hydroxylation was biphasic, the calculated low K_m being 75 μM and the high K_m being 253 μM. The substrate concentration range used for this kinetic study was from 40 to 800 μM. Liver microsomes from pyridine-treated male rats produced a K_m of 88 μM (results not shown). Consequently, P450 2E1 is probably the major enzyme contributing to the low K_m of 75 μM, and has a much greater affinity for CLZO than the second enzyme (high $K_m = 253$ μM). In rat liver microsomes, the values of the kinetic parameters (K_m) observed by Jayyosi et al. (1995) with a substrate concentration range of between 0.2 and 400 μM were 33 and 116 μM for low and high K_m, respectively. These authors observed, using liver microsomes from dexamethasone and isoniazid treated male rats, K_m values of 26 and 93 μM, similar to those observed in the control rats. Dexamethasone and isoniazid are inducers of hepatic P450 3A and 2E1, respectively. The K_m value of cytochrome P450 2E1 observed using liver microsomes from control rats is very different between the present (75 μM) and the previous results (116 μM) of Jayyosi et al. (1995). This difference is not clear, but may be due to variations in experimental procedure (e.g. substrate concentration range).

The PB used in our experiments increased the hydroxylase activities evaluated with CLZO and PNP in both the male and female rats, and confirms the results of Chen and Yang (1996) which demonstrated a slight increase in CLZO metabolism. PB is an inducer of hepatic cytochrome P450 2B1/2, and also increases the levels of P450 2A1, 2C6, 3A2 and 3A9 (Gonzalez and Gelboin, 1994; Mahnke et al., 1997). In fact, a cytochrome P450 3A isoenzyme may probably be involved in both these hydroxylation reactions. In the male rat, dexamethasone, an inducer of P450 3A, increases PNP hydroxylation activity (Zerilli et al., 1998) and CLZO hydroxylation activity at low (2 or 10 μM) substrate concentrations (Jayyosi et al., 1995; Zerilli et al., 1998). Dexamethasone did not increase CLZO hydroxylase activity at a high (400 μM) substrate concentration (Zerilli et al., 1998), whereas in the same conditions the present results indicated an increase in this activity by PB. In addition, PB is a more potent inducer of P450 3A9 than dexamethasone (Mahnke et al., 1997). Therefore, it cannot be excluded that a second form of P450 3A, namely isoenzyme 3A9, is probably involved in CLZO hydroxylation. In view of the high abundance of P450 3A family in the liver, and of the high V_m (1.64 nmol/mg protein min) (cf. Fig. 2), this second form (isoenzyme 3A9) could be the second enzyme observed in the Michaelis–Menten kinetic of the control rats, characterized by a K_m of 253 μM.

In addition, microsomal NADPH-P450 oxido-reductase is also induced by PB treatment (Shiraki and Guengerich, 1984). The induction of this oxido-reductase, which increases the rates of P450-dependent reactions, may also contribute to the increased rate of CLZO hydroxylase (Monostory and Vereczkey, 1994). Thus, both the present and previous results would suggest that other factors (P450 isoenzymes 2A1, 2C6, 3A2 and 3A9 and oxido-reductase) may also affect the rate of CLZO and PNP hydroxylation. However, worthy of note is that P450 2E1 plays a major role in CLZO and PNP hydroxylation in control rats.

The findings presented above suggest that in the males, the induction of PNP and CLZO hydroxylase activities observed with the P450 2E1 inducers were more significant than those observed in the females. On the others hand, the activity in control rats and the induction of P450 2E1 can be monitored by PNP and CLZO hydroxylase activities, although neither of these two reactions is specifically catalysed by P450 2E1. CLZO can be hydroxylated by at least three or four different forms of cytochrome P450 (2E1, 1A, 3A), and PNP by at least two forms of cytochrome P450 (2E1 and 3A). In conclusion, PNP may be considered as the most specific, but this method is less sensitive. In view: (1) of the results observed in male rats; (2) of the PNP hydroxylase activity level of control rats; (3) of the significant increases in PNP hydroxylase activity observed in 7 week old animals; and (4) of the inductions observed with the P 450 2E1 inducers in function of sex, these results suggest the use of male rats aged 7 weeks as experimental models to study the role of cytochrome P450 2E1 in any future studies on the hepatotoxicity of low molecular weight industrial chemicals.

Acknowledgements

The authors wish to express their thanks to Mrs Roussel for her secretarial assistance.

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Toxicology Letters 106 (1999) 181–190

Toxicology Letters

Absence of neurovisual effects due to tissue and blood cholinesterase depression in a chronic disulfoton feeding study in dogs

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Received 4 January 1999; accepted 4 March 1999

Abstract

Technical grade disulfoton (DiSyston) was fed to Beagle dogs (four animals per sex and treatment level) at nominal concentrations of 0, 0.5, 4 and 12 ppm for 1 year. The purpose of this study was to characterize the potential general and neurovisual toxicity according to routine Environmental Protection Agency (EPA) guideline requirements, and by use of ancillary ocular and neurologic tests established in this Laboratory. Ophthalmological tests included: ocular tissue cholinesterase and histopathology, electroretinography (ERG), tracking, refractivity, intraocular pressure and pachymetry (corneal thickness) measurements. Neurological examinations included; peripheral and cranial reflex tests, task performance tests, gait and behavioral observations, and rectal temperature measurements. Plasma, erythrocyte and corneal cholinesterase were significantly depressed at 4 and 12 ppm in both sexes. Brain cholinesterase was depressed at 4 and 12 ppm in females. Retinal cholinesterase was depressed at 4 ppm in females and at 12 ppm in males. Ciliary body cholinesterase was depressed at 12 ppm in both sexes. Despite these cholinergic effects, there were no ophthalmologic findings in measurements of ERG, tracking, refractivity, intraocular pressure or pachymetry. There were no clinical neurology findings related to compound administration. We conclude that 0.5 ppm was a no-observable effect level (NOEL), and effects were limited to cholinesterase changes that had no detectable physiologic impact. This study demonstrates that special mechanistic investigations incorporated within guideline studies, enhances scientific integrity and can minimize the need for dedicated organ system studies. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Disulfoton; Cholinesterase; Electroretinography; Neurovisual toxicity

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PII: S0378-4274(99)00064-8

1. Introduction

The concern regarding neurovisual effects and organophosphate exposure was based on reports of eye disease in children from the Saku agricultural region of Japan who were exposed to field spraying of high doses of several chemicals during the 1950s and 1960s (Plestina and Piukovic-Pelestina, 1978). Since that time, ocular effects testing has been conducted on organophosphates at significant levels of cholinesterase depression, with varying results. For example, fenthion was found to cause retinal degeneration in the rat (Imai et al., 1983), while ethyl parathion did not produce functional or histopathological changes in beagle dogs (Atkinson et al., 1994). It is known that the potential for ocular toxicity involves a wide range of compounds such as methanol, chloroquine, phenothiazines, indomethacin, sparsomycin, as well as, certain organophosphates (Potts, 1986). The purpose of conducting electroretinographic (ERG) measurements of wave component amplitudes and latencies was to evaluate disulfoton (ethylthiometon, Di-syston), using the most current toxicological methods for detecting the potential for ocular toxicity. Previously, morphologic changes in the extraocular muscles, optic nerve and sural nerve had been reported in mongrel dogs given disulfoton by capsule at doses of 0.5, 1 and 1.5 mg/kg per Da for 2 years (Mukuno et al., 1973). Disulfoton (*O,O*-diethyl *S*-[2-(ethylthio)ethyl]phosphorodithioate) is an organophosphate insecticide used to enhance the production of ornamental, vegetable and cotton crops.

The beagle dog is an appropriate animal model for organophosphate ocular toxicity because of its pigmentation, limited breed-associated genetic retinal dystrophies, and because of previous studies demonstrating xenobiotic-induced retinal toxicities similar to those in humans (Ishikawa, 1971; Hikita et al., 1973; Saito and Kubo, 1972; Suzuki and Ishikawa, 1974). Albino rodents have certain limitations as a model for human ocular toxicity, since they lack a pigmented retinal epithelium, have a high incidence of light-induced retinopathy, and there are strain differences from pigmented animals with respect to biochemical, physiological and pharmacological parameters (Creel, 1980; Boyes and Dyer, 1983). Albino strains have been found to have differences in metabolic biotransformations of xenobiotics and melanin-dependent physiological processes, and an absence of associated gene loci responsible for certain liver and kidney enzymes (Gibson et al., 1982; Jori et al., 1972).

Electroretinography and neurological examination in conjunction with associated biomarkers of activity, has been found to be a useful tool for evaluating ocular function when evaluating the toxicity of certain chemicals, drugs of abuse and pharmaceuticals (Jones et al., 1995; Roy et al., 1996; Mochizuki et al., 1991). The ERG wave results from high intensity light flashes and is composed of a rod–cone photoreceptor response (a-wave), a Muller and inner retinal cell response (b-wave), and a slow pigment epithelial depolarization response (c-wave). The oscillatory potentials are low amplitude wavelets produced by postsynaptic neuronal components, superimposed on the b-wave. Other tests that have been used to assess ocular function include: ophthalmic examination, intraocular pressure, pachymetery (corneal thickness), refractivity, ocular tissue cholinesterase levels and tracking. Histotechnology techniques have been extensively investigated for the demonstration and visualization of micropathologic ocular lesions, with particular attention given to the detection of organophosphate effects. (Render, 1990).

2. Materials and methods

2.1. Chemicals and substrates

Technical grade disulfoton (Batch No. 1085048/1030166) and an analytical standard were provided by Bayer AG, with structure and purity confirmed by nuclear magnetic resonance (NMR) and gas chromatography–mass spectroscopy (GC–MS). Corn oil at 1% of diet and a small amount of acetone rinse were used as the vehicle to dissolve the test substance. Corn oil and acetone used for mixing disulfoton with feed were purchased from Hazleton Laboratoriess

(Madison, WI) and Baxter Burdick Jackson (McGaw Park, IL), respectively. The oil was analyzed for contaminants by Corning–Hazelton (Hazleton, WI). The ration fed was Purina Mills Laboratories Canine Diet 5006-3 and was analyzed for contaminants by PMI Feeds, (St. Louis, MO). All feed mixtures were prepared weekly and stored under freezer conditions until fed.

2.2. Experimental animals

Purebred male and female (nulliparous and nonpregnant) Beagle dogs, *Canis familiaris*, were obtained from White Eagle laboratories (Doylestown, PA). The study dosing required 32 animals (16 males and 16 females) which were between 4 and 6 months of age when placed on study. The Beagle dog was selected as the test species because of its acceptance as the non-rodent species of choice for regulatory testing, the availability of a large historical database on the strain, and previous reports of histopathological and ERG abnormalities with disulfoton in the dog. Upon receipt, animals were given complete physical examinations by a veterinarian, placed into individual pens and then acclimated and quarantined for 7 days prior to pre-treatment measurements. The animals were housed in an accredited facility where, the environment was regulated and continuously monitored to maintain a room temperature range of 18–29°C , a relative humidity range of 30–70% and a daily photoperiod of approximately 12 h light and 12 h darkness.

2.3. Experimental design

The probable route of human exposure is via ingestion of foodstuffs or from accidental ingestion during manufacture or use, therefore formulation with feed was the desired method of delivery to assess the toxicodynamics of this compound. The nominal concentrations chosen were 0 (concurrent vehicle control), 0.5, 4, and 12 ppm disulfoton mixed in the feed on the basis of the active ingredient. Selection of these concentrations was based on previous dog studies, extrapolation of toxicokinetic cholinesterase and biotransformation data, and consideration of physiologic stress of anesthesia while performing ERGs. Animals were randomly allocated to study groups according to body weight and pre-treatment cholinesterase means using a computerized co-stratification procedure.

2.4. Analytical chemistry

Disulfoton concentrations in the diet of each batch prepared for the doses being tested were determined by GC analysis. Dietary batches of each concentration were measured for homogeneity by sampling the top, middle and bottom layers of the mixing bowl and determining the mean, standard deviation and coefficient of variation for each concentration. Stability of disulfoton mixed with dog ration was assessed in a previously developed validation method under freezer conditions (-23°C) for 28 days and at room temperature (22°C) for 14 days at concentrations of 0.25 and 25 ppm, which bracketed the test concentrations.

2.5. Cholinesterase and micropathology

Clinical chemistries and a complete blood count, including differentials, were performed on all animals at least twice prior to administration of disulfoton. One pre-treatment urinalysis was conducted. Blood was collected by cephalic venipuncture and voided urine was collected from stainless steel flooring. Two pre-treatment plasma (PChe) and erythrocyte (RChe) cholinesterase measurements were taken. Following initiation of dosing, hematological, clinical chemistry and urinalysis data were collected on all animals after approximately 3, 6, 9 and 12 (study termination) months on study. An additional 1 week plasma and erythrocyte cholinesterase measurement was done. Cholinesterase activity levels were measured in ocular tissues (retina, cornea, ciliary body) and extraocular muscles (lateral and dorsal recti) of the left eye. Plasma, erythrocyte, tissue and brain cholinesterase activity were determined using a modification of the Ellman reference method (Ellman et al., 1961) with acetylthiocholine as substrate and 6,6'-dithiodinicotinic acid as the

coupling agent (Hackathorn et al., 1983). Representative sections of the complete tissue list required for a guideline study were processed for all animals, stained with hematoxylin and eosin (H & E), and examined under the light microscope. The right eye, dorsal and lateral rectus muscles, and both optic nerves were preserved in Universal fixative for light microscopy and possible electron microscopy. The left eye, dorsal and lateral rectus muscles were frozen for cholinesterase analysis and therefore not examined microscopically. Previous validation work was used as justification for the fixative and processing method.

2.6. Ophthalmic examinations

Complete ophthalmic examinations (including tracking, refractivity, intraocular pressure and pachymetry) were conducted pre-treatment, at 6 months, and just prior to termination. A gross examination of the conjunctiva and pupillary response was performed with a transilluminator. After pupillary dilation with a mydriatic agent, the cornea, aqueous humor and lens were examined with a slit lamp microscope. Examinations of vitreous humor, retina, choroid and optic disc were performed with an indirect ophthalmoscope and condensing lenses. Photographs of the fundus were taken with a fundus camera. The examination was performed in a semi-darkened room.

2.7. Electroretinography

Electroretinography was performed at pre-treatment, at 6 months and just prior to sacrifice, to detect possible subclinical retinal changes that may have involved cells or certain component functions of the retina. A standard protocol for canine electroretinograms was used (Jones et al., 1994), which had been validated prior to use in this study with reference to the Good Automated Laboratory Practices (Jones et al., 1997). This five step ERG protocol consisted of: Step 1, (scotopic, single flash, Blue 10 dB) for rod response; Step 2, (scotopic, single flash) for maximal response; Step 3, (scotopic, single flash) for oscillatory potentials; Step 4, (photopic, single flash) for cone response; and Step 5, (photopic, 30 Hz flicker) for flicker cone response. The photopic stimulus was provided by a stroboscopic flash (Ganzfeld bowl) triggered and recorded by an electrodiagnostic system (LKC Technologies). The dogs were dark-adapted, following instillation of a mydriatic agent to promote pupil dilation. Only a faint red light was used to aid anesthesia and ERG procedures. Anesthesia was induced by mask using a semi-closed system with flow rates of 1 l/min of oxygen and 1 l/min of nitrous oxide, plus isoflurane. The depth of anesthesia was carefully controlled while the head was placed in a luminescent globe, with the dog in sternal recumbency on a foam surface. Artificial tears (0.5% methyl cellulose solution) were placed in the contact lens electrodes, which were applied to the center of the right and left cornea. The common negative needle electrode was placed in the intermaxillary region beneath the upper lip, and a single needle ground electrode was placed at the occipital crest. The average spectrum of the left and right eyes were computed to determine the a- and b-wave amplitudes and latencies. The analysis of the oscillatory potential positive peaks used an algorithm to filter out background noise from the wavelet components. The 30 Hz flicker response amplitudes and implicit times were analyzed using discrete Fourier transformations.

2.8. Neurologic examinations

A veterinary neurological examination was conducted pre-treatment, at 6 months and just prior to sacrifice to detect abnormalities through a series of reflex and reaction tests. Simple, inexpensive tools were used to conduct the examination; reflex hammer, strong pen light, and a toy ball. The general behavior, posture, gait, cranial and spinal nerve tests were scored as to severity of hypo- or hyper-reflexivity, presence or absence of signs, and abnormal response to task performance.

2.9. Statistical analysis

Continuous data that were examined statistically were analyzed initially by ANOVA to deter-

mine if statistical differences existed among the groups tested. If significance was suggested, each treatment group was compared to the control using a Dunnett's t-test. Confidence intervals at 95% were utilized to assess biological significance. Statistical analysis of the plasma and erythrocyte cholinesterase data were performed in two ways. The first statistical method derived a cholinesterase percent difference compared to the pre-treatment mean for each dog. A group mean was then calculated and statistical comparisons made between control and treated groups at each time interval. The second statistical method did not take into consideration each dog's pre-treatment baseline cholinesterase level, but rather, compared the treated animals' actual cholinesterase levels with those of the concurrent control dogs at each specific time interval. The advantage of comparison with pre-treatment means is that the influences of individual dog variability and maturation idiosyncrasies are reduced as confounding variables, and have less bias upon the toxicodynamic effects measured during the study. ANOVA followed by a Dunnett's t-test was used for these statistical comparisons. Brain and ocular cholinesterase data were analyzed by ANOVA followed by a Student's t-test to allow for other pairwise comparisons. Frequency data that were examined statistically were analyzed initially by a χ^2 procedure to determine if incidences differ statistically among the groups tested. If significance was suggested, each treatment group was compared to the control using a Fisher's Exact test. A probability value of $P < 0.05$ was accepted as significant.

3. Results

3.1. Analytical chemistry

Analytical data confirmed the compound to be homogeneously mixed and appropriately stable for all dose levels tested. The average daily consumption of disulfoton active ingredient in the male dose groups was 0, 0.015, 0.121 and 0.321 mg/kg per day, while the average daily consumption of active ingredient in the female dose groups was 0, 0.013, 0.094 and 0.283 mg/kg per day, corresponding to the nominal dietary concentrations of 0 (control), 0.5, 4 and 12 ppm.

3.2. Depression of plasma and erythrocyte cholinesterases

PChe was significantly decreased from concurrent control levels at nearly all treatment intervals in animals of the 4 and 12 ppm dietary groups in both sexes (Fig. 1a and b). The depression was slightly less severe in females when viewed as a percentage change from pretreatment values. Male %PChe means were also significantly different in the 0.5 ppm dose group at two intervals: at 3 months, where PChe was increased 1.3% from pretreatment levels and at 6 months, where PChe was decreased 10.2% from pretreatment. Even though male and female %PChe means were significantly different in the 0.5 ppm dose group at certain intervals, these were not considered biologically significant and did not persist through out the study. PChe was significantly decreased from concurrent control levels at nearly all intervals in both sexes in the 4 and 12 ppm dose groups.

Erythrocyte cholinesterase (RChe) was not significantly different from concurrent control levels in both sexes at the 0.5 ppm level (Fig. 2a and b). However, there was significant and/or biologically meaningful depression at most intervals in both sexes in the 4 and 12 ppm dose groups. RChe was significantly decreased from concurrent control levels at most treatment intervals in animals of both sexes in the 12 ppm groups. On a percentage of pretreatment basis, the depression was significant at all treatment intervals in the 12 ppm group, both sexes. At 4 ppm, female concurrent RChe was significantly depressed from 3 months to termination. When data were examined on a percentage of pretreatment basis, 4 ppm males also showed significant decreases at 3, 6, and 12 months, while females were confirmed by this examination to have significant depression at 3 and 6 months only. This statistical evidence is strong enough to allow interpretation of an RChe treatment effect at 4 ppm in both sexes.

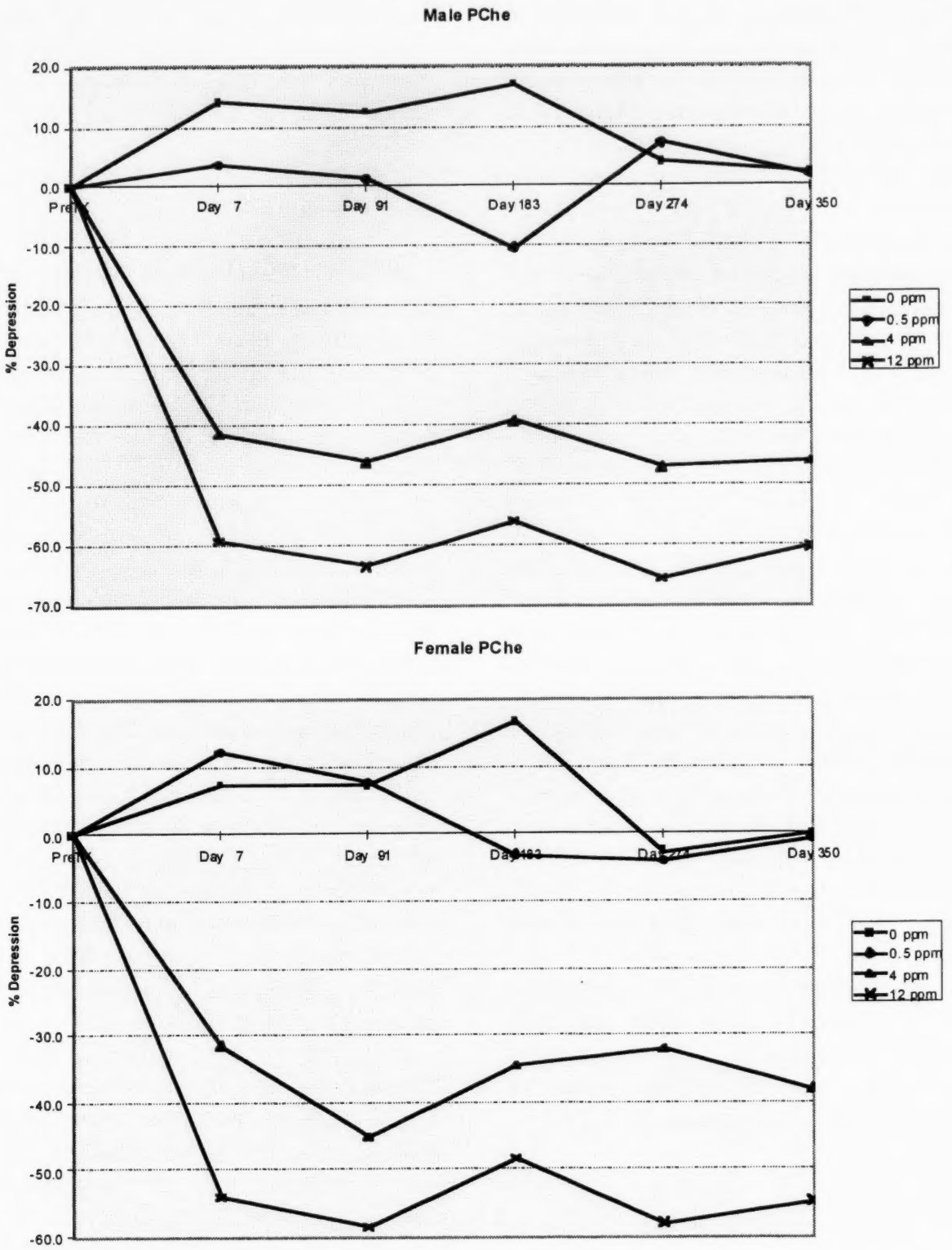

Fig. 1. Mean %PChe difference inhibition levels (top) compared to pre-treatment means in the male dogs treated with disulfoton for 1 year. (bottom). Mean %PChe difference inhibition levels compared to pre-treatment means in the female dogs treated with disulfoton for one year. In both sexes, the 4 and 12 ppm dose groups were statistically different from the corresponding time-point control group ($P < 0.05$) by ANOVA and Dunnett's t-test.

3.3. Depression of brain and ocular tissue cholinesterase

The mean brain cholinesterase data are presented in Table 1 for males and females. Brain cholinesterase (BChe) was significantly depressed from control levels in 4 and 12 ppm females only. Despite this amount of acetylcholinesterase de-

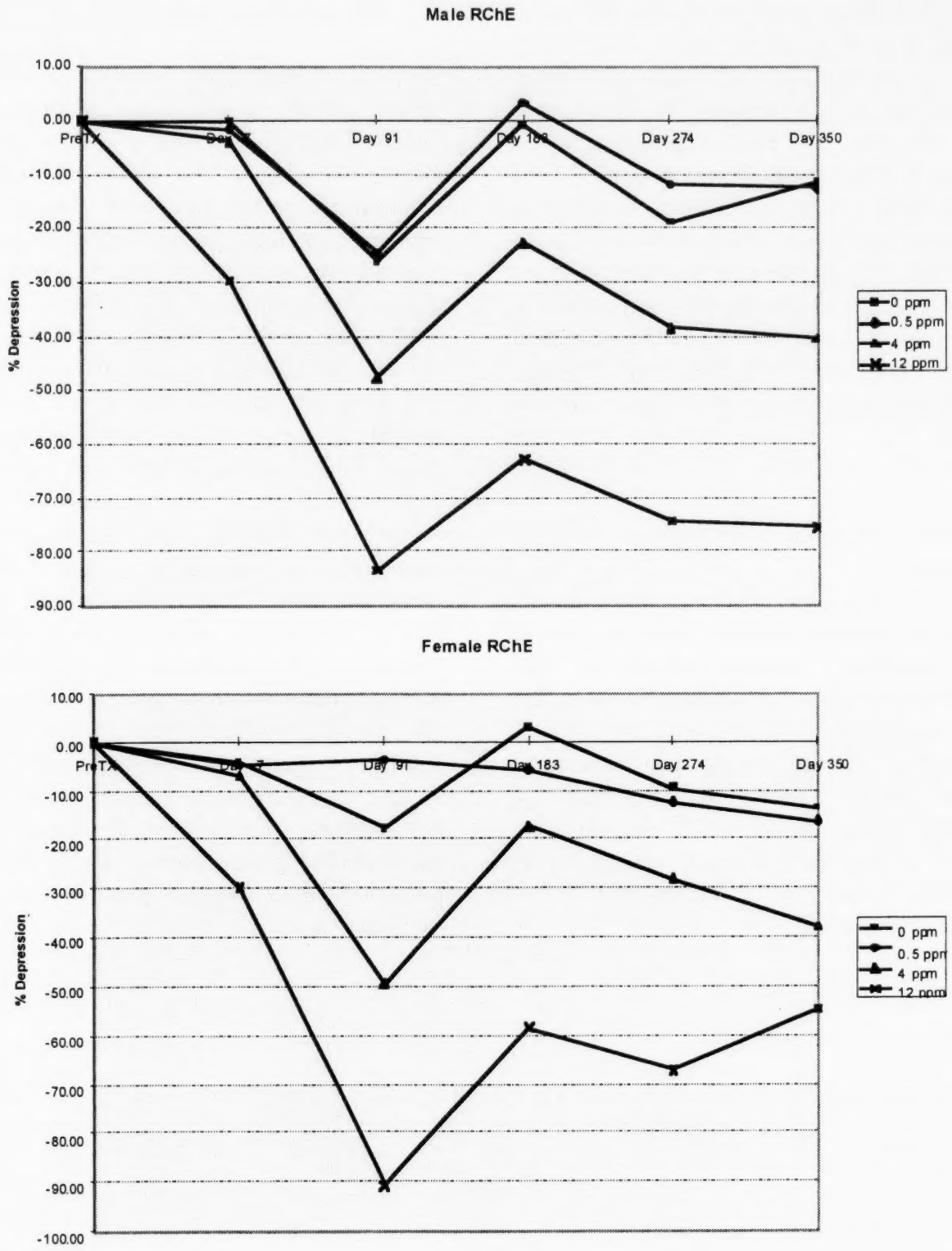

Fig. 2. Mean %RChe differences (top) compared to pre-treatment means in the male dogs treated with disulfoton for 1 year.(bottom). Mean %RChe differences compared to pre-treatment means in the female dogs treated with disulfoton for 1 year. In both sexes, the 4 and 12 ppm dose groups were statistically different from the corresponding time-point control group ($P < 0.05$) by ANOVA and Dunnett's t-test.

pression, there were no clinical neurology findings related to chronic disulfoton administration in this study.

The mean ocular tissue cholinesterase data are presented in Table 1 for the males and females. There were no significant differences in cholinesterase levels in lateral (LRM) or dorsal (DRM) rectal muscles. Cornea cholinesterase was significantly depressed in 4 and 12 ppm groups and in 0.5 ppm males. The control and 0.5 ppm

mean values for females were almost identical. Since females were, in general, more sensitive to cholinesterase depression in this study, and given the lack of significant depression in other areas of the eye at 0.5 ppm, the male depression noted was equivocal. Therefore, it was not noted to be a treatment-related effect. Retinal cholinesterase was significantly depressed from control in the 12 ppm males and 4 ppm female groups. Ciliary body cholinesterase was significantly depressed at 12 ppm animals in both sexes and significantly increased from control in the 0.5 ppm females. The latter change was considered to be a statistical aberration.

3.4. Ophthalmic, neurologic examinations and clinical chemistry

There were no ophthalmologic findings related to chronic disulfoton administration in this study, including tracking, refractivity, intraocular pressure and pachymetry. There were no clinical neurology findings related to chronic disulfoton administration detected during task performance or examination. There were no clinical chemistry, hematology or urinalysis findings in either sex at the dose levels tested, with the exception of cholinesterase depression.

3.5. Electroretinography

A set of waveforms (rod, cone, oscillatory potential, stroboscopic cone response) were collected according to a five step protocol from each eye (Jones et al., 1994). A previous validation study performed with similar sample size was able to detect statistical differences and biologically meaningful trends due to cholinesterase depression (Jones et al., 1995). There were no ERG changes in amplitude or latency related to chronic disulfoton administration that achieved the level of acetylcholinesterase depression observed in the ocular tissues and brain of these dogs. The negative a-wave and positive b-wave potentials found by stimulating the rod response at the pretreatment, 6 month interim and pre-termination intervals during this study found no dose–response or temporal effect in either sex due to disulfoton administration. The 5 wavelet oscillatory potentials did not exhibit a treatment related effect in amplitude or latency. Likewise, the a- and b-wave mean values for the cone response found no treatment or temporal response detected as being different from control in either sex. There was no change in amplitude or implicit time as measured by the flicker cone response that was statistical or biologically meaningful.

Table 1
Mean brain and ocular tissue %cholinesterase depression in the chronic disulfoton study

	Brain	LRM	DRM	Cornea	Retina	Cil. body
Males						
0 ppm	0.00	0.00	0.00	0.00	0.00	0.00
0.5 ppm	1.61	−14.29	−16.67	−33.33*	0.00	−15.38
4 ppm	−12.90	−14.29	0.00	−50.00*	−33.33	−23.08
12 ppm	−32.26	−14.29	−16.67	−66.67*	−66.67*	−53.85*
Females						
0 ppm	0.00	0.00	0.00	0.00	0.00	0.00
0.5 ppm	1.59	−14.29	−14.29	0.00	25.00	36.36*
4 ppm	−22.22*	14.29	0.00	−60.00*	−25.00	−18.18
12 ppm	−33.33*	−14.29	−28.57	−60.00*	−50.00*	−45.45*

* Statistically different from concurrent control ($P<0.05$) by ANOVA and Student's t-test. Values are % differences from corresponding controls with; LRM, lateral rectus muscle; DRM, dorsal rectus muscle; Cil. body, ciliary body

4. Discussion

A component analysis approach of the ERG waveform was used to better identify potential effects of disulfoton administration. The a-wave is the fast negative photoreceptor response, while the b- and c-waves originate primarily from the Muller and pigment epithelial cells (Masland and Ames, 1976). The peak amplitudes and latencies of the a- and b-waves are the primary parameters used to study retinal responses, while the c-wave is not usually measured. The flicker response was included, since it is reported to be a test which is relatively insensitive to recording interferences (Severns et al., 1991). Electroretinography in the rat model has detected ocular effects from certain organophosphates. Intramuscular injection of an organophosphate in Wistar rats caused shortening of the latency time and an increase in the amplitude of the a-wave (Imai, 1974), however at doses 5 times greater the a- and b-wave amplitudes decreased. In Wistar rats treated with chlorpyrifos, a decrease in ERG a- and b-wave amplitudes of more than 50% and increased latencies of 10–20% were associated with decreased plasma, erythrocyte and brain cholinesterase levels (Yoshikawa et al., 1990).

The dog has been reported to demonstrate ocular toxicity due to organophosphate exposure (Hikita et al., 1973; Suzuki and Ishikawa, 1974), including a higher-dose disulfoton capsule study (Uga et al., 1977). We had previously demonstrated that ERG rod and cone amplitude depressions occur in the dog due to cholinesterase inhibition, and that this ERG methodology was capable of detecting treatment-related differences (Jones et al., 1995). However in this study, there were no ERG changes that were considered to be related to chronic disulfoton administration, suggesting that the levels of cholinesterase inhibition in the ocular tissues, brain, erythrocyte and plasma were not detrimental to electrophysiologic function. The most sensitive ocular tissues to cholinergic depression were the cornea, retina and ciliary body. This result was similar to a physostigmine ERG validation study, where the cornea and retina were inhibited 60 and 70%, respectively. This acute steady-state level of ocular tissue cholinergic depression in that study resulted in ERG changes of decreased rod b-wave amplitude, decreased cone b-wave amplitude and decreased flicker cone amplitude (Jones et al., 1995). The rod b-wave amplitude and flicker cone amplitude were decreased at all levels of cholinesterase depression, in a dose-related manner during multiple steady-state infusion that achieved %RChe inhibitions of 5, 25, 40 and 52 (Jones et al., 1995). Tolerance to disulfoton behavioral and neurochemical effects in rats has been attributed to muscarinic receptor down-regulation (Llorens et al., 1993), which could possibly explain the difference between acute and chronic neurovisual effects. In this chronic feeding study with disulfoton, there were no histological or gross pathology changes detected in the retina of any dose group, despite cholinesterase inhibitions of up to 67%. This chronic systemic cholinesterase inhibition in the brain (as much as 33%), cornea, retina and ciliary body did not appear to have altered the physiologic function of the visual system in spite of marked tissue cholinesterase inhibition. These results are in contrast to the morphologic changes described by the Mukuno study, which used a parenteral route of exposure at much higher doses in mongrel dogs (Mukuno et al., 1973). According to the degree of cholinesterase inhibition produced by the oral route, we believe the choice of disulfoton levels was realistic in terms of the disposition and metabolism capacities for the dogs in this study.

The neurologic examination of observations, reflexes and task performances did not detect function abnormalities at the levels of cholinesterase depression observed in this chronic disulfoton feeding study. This study demonstrates that special mechanistic investigations incorporated within guideline studies, enhance scientific integrity and can minimize the need for dedicated organ system studies. The interpretation of ancillary ophthalmic tests were aided by the battery of parameters measured in the ongoing regulatory study. This experimental design and approach was consistent with the Food Quality Protection Act directive for complete, sound reliable scientific data.

We conclude that the toxicodynamics of disulfoton at the levels tested involve cholinergic effects on the plasma, erthyron, brain and certain ocular tissues, and that compensatory responses of the animal allowed for clinical neurologic and visual homeostasis to be maintained.

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Toxicology Letters 106 (1999) 191–200

Toxicology Letters

Peroxidase-catalyzed in vitro formation of polychlorinated dibenzo-*p*-dioxins and dibenzofurans from chlorophenols

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Received 5 January 1999; received in revised form 10 March 1999; accepted 17 March 1999

Abstract

Chlorophenols (CP) are transformed in vitro to polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F) by a peroxidase-catalyzed oxidation. This is shown for 2,4,5-tri-, 2,3,4,6-tetra- and pentachlorophenol with plant horseradish peroxidase and with myeloperoxidase recovered from human leukocytes, each in the presence of hydrogen peroxide. The yield, the reaction and the PCDD/F-pattern found are dependent on the CP. The amounts of PCDD/F formed within 4 or 24 h are in the µmol/mol-range for all substrates and both peroxidases. The experiments suggest that biochemical formation of PCDD/F from precursors such as CPs can take place in the human body and that this metabolic pathway may lead to a higher inner exposure to PCDD/F than up to now assumed based on intake data for PCDD/F. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Peroxidase; Horseradish peroxidase; Myeloperoxidase; Hydrogen peroxide; Enzymatic formation; Polychlorinated dibenzo-*p*-dioxins; Polychlorinated dibenzofurans; Octachlorodibenzo-*p*-dioxin; 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; Chlorophenols; Pentachlorophenol; 2,3,4,6-Tetrachlorophenol; 2,4,5-Trichlorophenol

1. Introduction

Several mass balance studies on humans (Moser et al., 1996; Schrey et al., 1998) and studies on cattle, which were in contact or fed with pentachlorophenol (PCP) treated wood, (Fries et al., 1996; Fries et al. 1997) indicate a higher fecal excretion of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F), especially higher chlorinated PCDD, in relation to the amount ingested. The results point to the possible biochemical formation of PCDD/F in the organisms from precursors such as chlorophenols (CP) or the known contaminants of technical-grade CP,

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0378-4274/99/$ - see front matter © 1999 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0378-4274(99)00066-1

i.e. chlorinated phenoxyphenols. Biochemical formations of PCDD/F from chlorophenols and other precursors have been observed in sewage sludge (Öberg et al., 1992; Schramm et al., 1996), compost (Öberg et al., 1992, 1993; Schäfer et al., 1993), in in vitro studies catalyzed by horseradish peroxidase (HRP) (Svenson et al., 1989a; Svenson et al. 1989b; Öberg et al., 1990; Wagner et al., 1990), bovine lactoperoxidase (LP) (Öberg et al., 1990) or more complex enzymatic systems such as whey or culture filtrates from fungi (Wagner et al., 1990). The formation of octachlorodibenzo-*p*-dioxin from reagent or technical grade pentachlorophenol and from nonachloro-2-phenoxyphenol, a contaminant of technical PCP, has been observed in rats (Feil and Tiernan, 1997; Huwe et al., 1998).

Peroxidases are omnipresent bi-substrate enzymes in nature. Within the catalyzed reactions the presence of hydrogen peroxide is necessary because it is used as the electron accepting substrate. In the present study we investigated the in vitro formation of PCDD/F from the commercial significant CPs 2,4,5-trichlorophenol (2,4,5-TrCP), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP) and pentachlorophenol catalyzed by plant HRP and by myeloperoxidase (MYP) from human leukocytes, each in the presence of hydrogen peroxide.

In the present study the MYP catalyzed formation of PCDD/F from different chlorophenols, from chlorophenol substrate mixtures and a comparison with the catalyzation by HRP are described for the first time. The experiments with HRP were repeated here for better comparison because formations of PCDD/F might be influenced by impurities of the substrates.

2. Materials and methods

2.1. Enzymes and substrates

HRP Type II (P 8250/Lot 16H9522) was obtained from Sigma. MYP recovered from human leukocytes and lyophylized from 0.02 M sodium acetate buffer at pH 6.0 (MPO; EC 1.11.1.7, M 6908/Lot 126H9402) was purchased from Sigma. 2,4,5-TrCP (99.9% chemically pure, certified, Dr Ehrenstorfer) and 2,3,4,6-TeCP (97.2% chemically pure, certified, Dr Ehrenstorfer) were obtained from Promochem (Wesel, Germany) and PCP (certified reference material) was from International Physical Laboratory (UK). Methanolic solutions of the chlorophenols were made with concentrations of 2, 5 or 15 mg/ml.

2.2. Composition and incubation conditions of the experiments

The reactions with HRP were incubated over 24 h in a total volume of 10 ml of a dimethyl succinic acid (DSA) buffer at pH 4.0 at room temperature as described by Wagner et al. (1990). All experiments with myeloperoxidase were carried out in a potassium dihydrogen phosphate buffer (pH 5.4) with a total volume of 2.0 ml in 20 ml glass test tubes at 37°C. The incubation time was 4 h in these experiments. The incubation was started with the addition of hydrogen peroxide. All series were performed with two blank controls containing either the chlorophenol substrate or the peroxidase while all other additions were left unchanged. The detailed lists of composition are shown in Tables 1 and 2.

2.3. Analysis

After incubation 100 μl of a standard solution containing 17 $^{13}C_{12}$-labelled PCDD/F congeners (2.5 or 5.0 pg/μl) in toluene, 3 ml saturated ammonium sulphate solution and 3 ml ethanol were added to an 2-ml aliquot of the HRP incubations or to the total amount of the MYP incubations. The samples were extracted three times with 4 ml hexane. The hexane extracts were dried over sodium sulphate and purified using three column clean up steps: a combination of neutral, acidic and basic silica gels, alumina and activated charcoal (Nygren et al. 1988; Höckel and Hagenmaier, 1995). After addition of 2 μl dodecane as keeper the cleaned extracts were evaporated to dryness using a nitrogen stream and 10 μl toluene containing 2.5 pg/μl 1,2,3,4-[$^{13}C_{12}$] tetraCDD were added as recovery standard.

The analytical instrument system consisted of a VG AutoSpec high-resolution mass spectrometer and a Hewlett-Packard 5890 series II gas chromatograph equipped with a Gerstel KAS 2 vaporization system (MS-parameters: single ion recording mode; resolution 8000–10 000 at 10%; electron impact ionization at 40 eV; perfluorokerosene lock mass check; observation of two ions

Table 1
List of experiments with HRP (total volume: 10 ml)

Experiment No.	DSA buffer (mM)	HRP (U)[a]	CP (mM)	Hydrogen peroxide (mM)
H0 (CP-blank)	10	2.0	–	2.0
2,4,5-TrCP				
H1 (blank)	10	–	0.1 (2,4,5-TrCP)	2.0
H2	10	2.0	0.1 (2,4,5-TrCP)	2.0
2,3,4,6-TeCP				
H3 (blank)	10	–	0.1 (2,3,4,6-TeCP)	2.0
H4	10	2.0	0.1 (2,3,4,6-TeCP)	2.0
PCP				
H5 (blank)	10	–	0.1 (PCP)	2.0
H6	10	2.0	0.1 (PCP)	2.0

[a] Units as specified by the manufacturer.

Table 2
List of experiments with MYP (total volume: 2 ml)

Experiment No.	KH_2PO_4 buffer (mM)	MYP (U)[a]	CP (mM)	Hydrogen peroxide (mM)
M0 (CP-blank)	0.375	1.0	–	0.5
2,4,5-TrCP				
M1 (blank)	0.375	–	1.0 (2,4,5-TrCP)	1.0
M2	0.375	1.0	1.0 (2,4,5-TrCP)	1.0
M3	0.375	1.0	0.1 (2,4,5-TrCP)	0.1
2,3,4,6-TeCP				
M4 (blank)	0.375	–	1.0 (2,3,4,6-TeCP)	1.0
M5	0.375	1.0	1.0 (2,3,4,6-TeCP)	1.0
PCP				
M6 (blank)	0.375	–	1.0 (PCP)	1.0
M7	0.375	1.0	0.1 (PCP)	0.1
M8	0.375	1.0	1.0 (PCP)	0.5
M9	0.375	1.0	0.1 (PCP)	1.0
M10	0.375	1.0	1.0 (PCP)	1.0
M11	0.375	1.0	1.0 (PCP)	2.0
Equimolar multisubstrate experiments				
M12	0.375	1.0	1.0 (2,4,5-TrCP), 1.0 (PCP)	1.0
M13	0.375	1.0	0.1 (2,4,5-TrCP), 0.1 (PCP)	0.1
M14	0.375	1.0	1.0 (2,3,4,6-TeCP), 1.0 (PCP)	1.0
M15	0.375	1.0	0.1 (2,3,4,6-TeCP), 0.1 (PCP)	0.1
M16	0.375	1.0	1.0 (2,4,5-TrCP), 1.0 (2,3,4,6-TeCP), 1.0 (PCP)	1.0

[a] Units as specified by the manufacturer.

Table 3
HRP- or MYP-catalyzed formation of PCDD/F from PCP (detailed information on the experiments are given in Tables 1 and 2)

Experiment No.	OctaCDD (pg)	ΣPCDD/F (pg)	OCDD (μmol/mol $_{PCP}$)[a]	ΣPCDD/F (μmol/mol $_{PCP}$)[a]
H5 (bl)	64	150	–	–
H6	6800	6910	14.7	14.9
M6 (bl)	140	155	–	–
M7	1600	1660	15.9	16.4
M8	3500	3670	3.65	3.88
M9	1600	1600	15.9	16.0
M10	10 000	10 400	10.7	11.1
M11	6100	6350	6.48	6.80

[a] Blank (experiment H5 or M6) corrected values.

Table 4
HRP- or MYP-catalyzed formation of PCDD/F from 2,4,5-TrCP (detailed information on the experiments are given in Tables 1 and 2)

Experiment No.		PentaCDD	HexaCDD	HeptaCDD	PentaCDF	HexaCDF	ΣPCDD/F
H1 (bl)	pg	<0.5	<0.5	1.2	4.9	0.76	50.3
H2	pg	560	510	9.6	62	140	1340
	μmol/mol$_{TrCP}$[a]	1.57	1.30	0.02	0.17	0.37	3.45
M1 (bl)	pg	<0.5	9.2	17	< 0.5	1.9	83.2
M2	pg	150	140	74	89	310	782
	μmol/mol $_{TrCP}$[a]	0.21	0.17	0.07	0.13	0.41	0.99
M3	pg	24	30	7.4	18	53	138
	μmol/mol $_{TrCP}$[a]	0.34	0.27	0.00	0.26	0.68	1.55

[a] Blank (experiment H1 or M1) corrected values.

each for native and labelled isomers; setting of five time windows; GC-parameters: column: J&W Scientific, DB-5, 60 m, 0.1 μm film thickness; temperature program: 180°C (3 min), 5°C/min, 220°C (16 min), 5°C/min, 235°C (7 min), 5°C/min, 280°C (15 min); injector program: 60°C (60 s), 12°C/s, 330°C (10 min), split off (1 min); split on (2 min); injection volume: 4 μl).

The PCDD/F concentrations were calculated considering the recovery rates of the internal standard compounds and response factors.

3. Results and discussion

The results of the PCDD/F homologue groups of major interest and the sum of the PCDD/F are given in the Tables 3–6 for the experiments with PCP, 2,4,5-TrCP, 2,3,4,6-TeCP and the multisubstrate experiments.

The PCDD/F concentrations in the chlorophenol free blanks (exp. H0 and M0) were near to the detection limit for all congeners and very small amounts were detected in the HRP or MYP free blank samples (exp. H1, H3, H5, M1, M4 and M6). In all other samples a significant formation of PCDD/F—2,3,7,8-chlorosubstituted as well as non-2,3,7,8-chlorosubstituted congeners—was observed. The formation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin was only observed in the experiments using 2,4,5-TrCP.

3.1. Monosubstrate experiments with PCP

The monosubstrate experiments with PCP and both HRP or MYP showed a predominant formation of OctaCDD (Table 3). The amounts formed were in the same order of magnitude for both peroxidases. The amount of OctaCDD built by

MYP showed a tendency to increase with the amount of the substrate. But highest formations were observed with the lower PCP concentrations (exp. M7 and M9), whereas the hydrogen peroxide concentration only showed an influence on the PCDD/F formation in the higher dosed (1 mM) (exp. M8, M10, M11) but not in the lower dosed (0.1 mM) (exp. M7 and M9) experiments.

3.2. Monosubstrate experiments with 2,4,5-TrCP and 2,3,4,6-TeCP

In comparison to the experiments with PCP the formation of PCDD/F by HRP or MYP was lower in the monosubstrate experiments with 2,4,5-TrCP (Table 4) and 2,3,4,6-TeCP (Table 5) and showed different homologue patterns. The main homologue groups were the PentaCDD, followed by HexaCDD and HexaCDF (2,4,5-TrCP, HRP), the HexaCDF, PentaCDD, HexaCDD, and PentaCDF (2,4,5-TrCP, MYP), the OctaCDD, HeptaCDD and HexaCDD (2,3,4,6-TeCP, HRP) or the HeptaCDD, OctaCDD and HexaCDD (2,3,4,6-TeCP, MYP). A comparison of the amounts of PCDD/F formed on a molar basis for the HRP catalyzed monosubstrate experiments with 0.1 mM concentrations (exp. H2, H4 and H6) is given in Fig. 1 and for the MYP catalyzed monosubstrate experiments with 1 mM concentrations (exp. M2, M5 and M10) in Fig. 2.

Table 5
HPR- or MYP-catalyzed formation of PCDD/F from 2,3,4,6-TeCP (detailed information on the experiments are given in Tables 1 and 2)

Experiment No.		HexaCDD	HeptaCDD	OctaCDD	ΣPCDD/F
H3 (bl)	pg	2.7	14	23	189
H4	pg	43	180	2000	2530
	$\mu mol/mol_{TeCP}$[a]	0.10	0.39	4.30	5.12
M4 (bl)	pg	17	220	65	1320
M5	pg	260	2200	1100	4610
	$\mu mol/mol_{TeCP}$[a]	0.31	2.33	1.13	3.77

[a] Blank (experiment H3 or M4) corrected values.

Table 6
MYP-catalyzed formation of PCDD/F from CP mixtures (detailed information on the experiments are given in Table 2)

Experiment No.		PentaCDD	HexaCDD	HeptaCDD	OctaCDD	HexaCDF	ΣPCDD/F
M12	pg	280	680	13 000	1400	880	16 000
	$\mu mol/mol_{\Sigma CP}$	0.20	0.43	7.64	0.76	0.59	9.70
M13	pg	120	100	1300	140	200	1830
	$\mu mol/mol_{\Sigma CP}$	0.84	0.64	7.64	0.76	1.33	11.3
M14	pg	12	150	3800	9700	450	14 800
	$\mu mol/mol_{\Sigma CP}$	0.01	0.10	2.23	5.27	0.30	8.34
M15	pg	0.29	7.0	84	310	29	492
	$\mu mol/mol_{\Sigma CP}$	0.00	0.04	0.49	1.69	0.19	2.78
M16	pg	36	1100	3000	910	890	6760
	$\mu mol/mol_{\Sigma CP}$	0.02	0.47	1.18	0.33	0.40	2.70

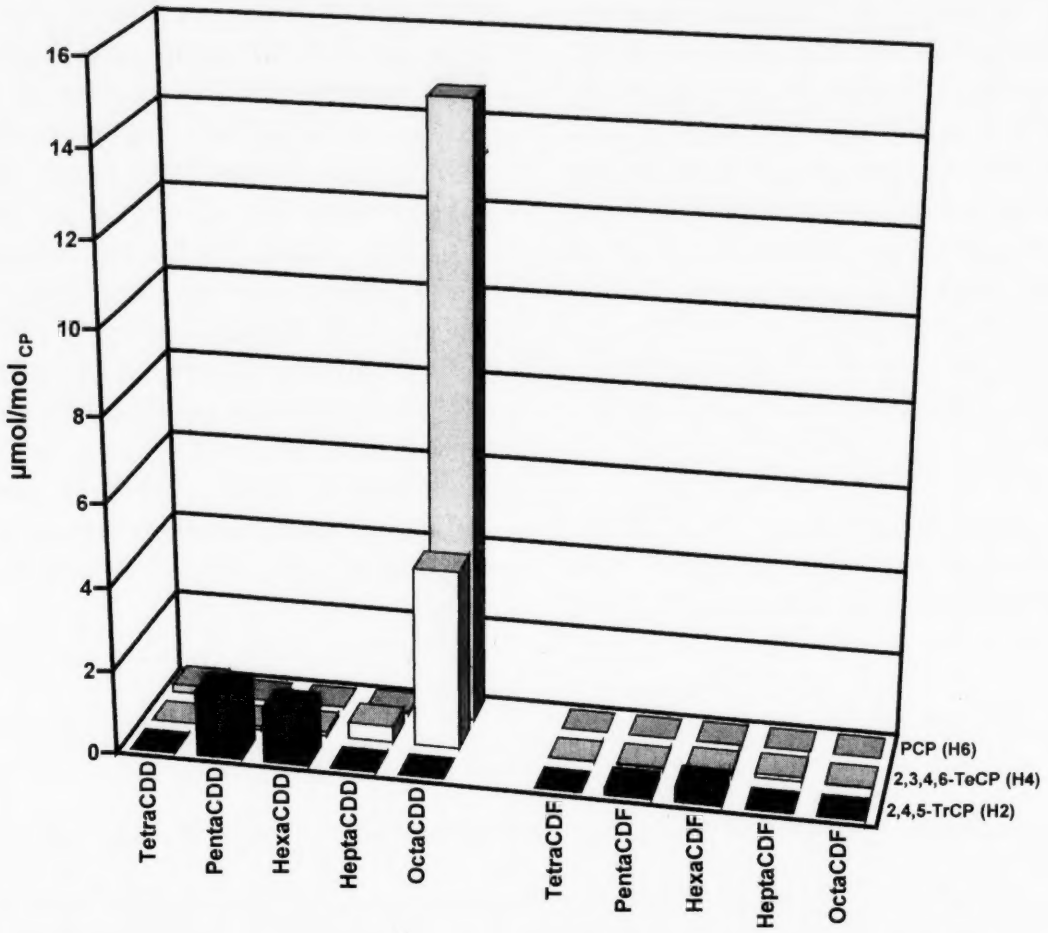

Fig. 1. PCDD/F formation of the 0.1 mM monosubstrate experiments H2, H4 and H6 with HRP (detailed information on the experiments are given in Table 1)

3.3. MYP-catalyzed multisubstrate experiments with mixtures of 2,4,5-TrCP, 2,3,4,6-TeCP and PCP

Within the MYP catalyzed experiments series (Table 6) the combination of equimolar mixtures of 2,4,5-TrCP and PCP resulted in the formation of HeptaCDD as the major component, whereas OctaCDD, followed by HeptaCDD, were the main components in the experiments with equimolar mixtures of 2,3,4,6-TeCP and PCP. Hepta- and HexaCDD were the main components in the experiment containing all three chlorophenols. The results of the experiments M12, M14 and M16 are shown in Fig. 3.

3.4. Comparison with other in vitro studies

A comparison of the experiments with HRP and MYP with studies of other authors is given in Table 7. All experiments with PCP gave OctaCDD as the main component. The formations observed in the studies with LP and HRP published by Öberg et al. (1990) and Öberg and Rappe (1992) were two to four times higher as in our own experiments with HRP and MYP showing both about 15 $\mu mol/mol_{PCP}$. The PCDD/F pattern found in the experiments with 2,4,5-TrCP is different between authors. The differences observed could be an effect of the experimental conditions, especially the different peroxidase and hydrogen peroxide concentrations used. In addition the discrepancies may be attributed to different analytical procedures and to different quality grades of the CPs used.

3.5. Comparison with in vivo studies

First in vivo studies with rats fed with 0.1 mg/day of purified PCP over 14 days showed no higher PCDD/F levels in the liver in comparison to a control group. Administration of reagent

grade PCP and technical grade PCP showed the formation of higher chlorinated PCDD (Feil and Tiernan, 1997). The main component was OctaCDD with levels 2.6 or 1042 times higher in comparison to the control group. In other experiments the formation of OctaCDD from nonachloro-2-phenoxyphenol, which is a known contaminant of technical PCP, was observed in rats (Huwe et al., 1998). Both studies confirm the formation of PCDD/F in vivo from PCP impurities.

3.6. Assessment and conclusion for human exposure

All observed formations in the in vitro studies are in the μmol/mol range and so—at first glance—they seem to be of minor importance, but the estimated intake of pentachlorophenol for unexposed persons in Germany is in the range of 1–2 μg/day (Butte and Heinzow, 1995; HBM-Kommission, 1997) and in the mass balance studies on humans (Schrey et al., 1998) the fecal excretion of OCDD of adults was on average 2300 pg/day higher than the dietary intake. Chlorophenols can also be significant metabolites of other chlorinated substances such as chlorobenzenes and chlorocyclohexanes. Typical serum concentrations of pentachlorophenol for unexposed persons from Germany range up to 20 μg/l and occupational exposed individuals show CP concentrations 10 to 1,000 times higher (HBM-Kommission, 1997). From this background and under consideration of the observed amounts formed in the in vitro experiments additional PCDD/F formation in the pg/day range would be expected, although kinetic data are not available at the moment.

It should be noted, that in all cases the presence of hydrogen peroxide as second substrate is neces-

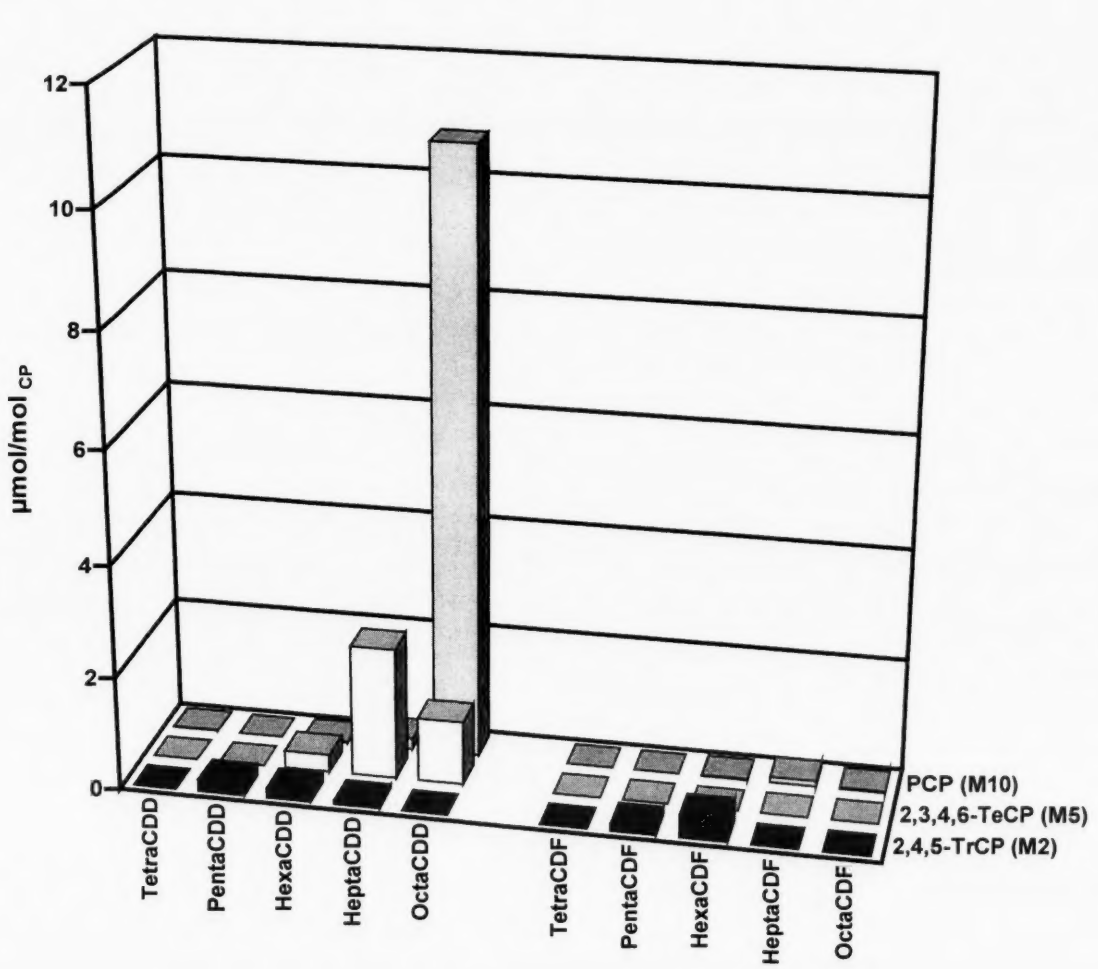

Fig. 2. PCDD/F formation of the 1 mM monosubstrate experiments M2, M5 and M10 with MYP (detailed information on the experiments are given in Table 2)

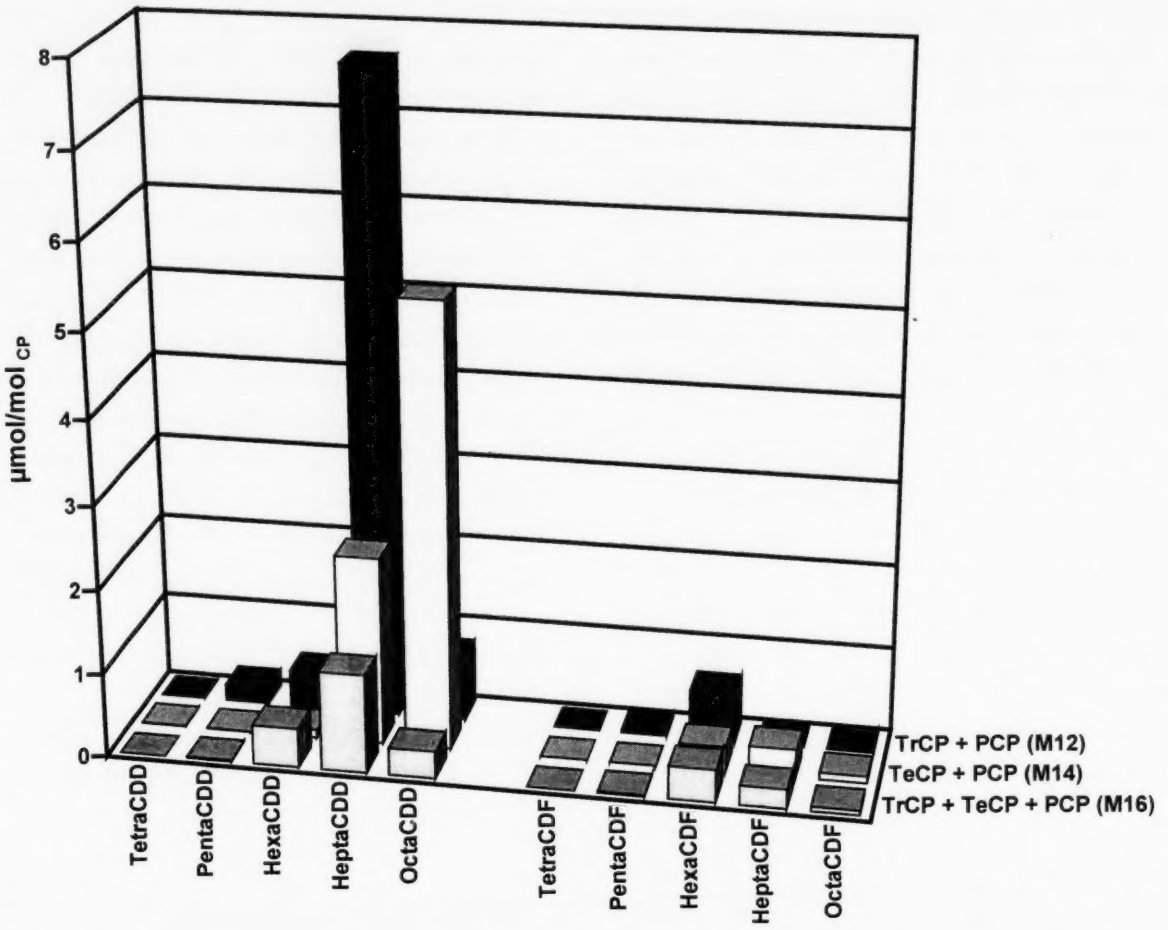

Fig. 3. PCDD/F formation of the multisubstrate experiments M12, M14 and M16 with MYP (detailed information on the experiments are given in Table 2)

Table 7
Comparison with data from literature

Parameter	Öberg et al. (1990) and Öberg and Rappe (1992)		Svenson et al. (1989b) HRP	This study	
	LP	HRP	HRP	HRP	MYP
Experimental conditions					
2,4,5-TrCP (mM)	0.1	0.1	0.1	0.1	0.1
PCP (mM)	0.1	0.1	–	0.1	0.1
Peroxidase (U/ml)	1.3–1.7	1.3–1.7	1.3–1.7	0.2	0.5
Hydrogen peroxide (mM)	0.2	0.2	9.0	2.0	0.1
Formation of PCDD/F from 2,4,5-TriCP[a]					
PentaCDD (µmol/mol $_{TrCP}$)	3.60	1.27	3.37	1.57	0.34
HexaCDD (µmol/mol $_{TrCP}$)	1.01	0.86	5.88	1.30	0.27
PentaCDF (µmol/mol $_{TrCP}$)	0.37	0.06	0.37	0.17	0.26
HexaCDF (µmol/mol $_{TrCP}$)	0.04	0.03	0.75	0.37	0.68
ΣPCDD/F (µmol/mol $_{TrCP}$)	5.73	2.43	10.5	3.45	1.55
Formation of PCDD/F from PCP					
OctaCDD (µmol/mol $_{PCP}$)	34.8	57.9	–	14.7	15.9

[a] Wagner et al. (1990) found a formation of 5,7 $\mu g_{\Sigma\ PCDD/F}/g_{2,4,5\text{-}TrCP}$. The formation on a molar basis cannot be calculated due to missing data for the several homologue groups.

sary. Cellular and subcellular hydrogen peroxide concentrations at steady state are in the range 1–100 nM (Chance et al., 1979). The formations of PCDD/F in the human body can first takeplace in the digestive tract, where peroxidases can be present as part of microorganisms, vegetable or animal food (Wagner et al., 1990). Anyway, different peroxidase systems can be found in many organs and cells. The MYP used in this study is a component of human neutrophile granulocytes. The content of MYP is up to $5\%_{\text{dry matter}}$ in the peripheral cells, whereas the content in growing granulocyte cells in bone marrow can be higher (Marquardt and Schäfer, 1994). This shows that there are many locations where the formation of PCDD/F by peroxidase systems can take place.

It must be mentioned that the CPs used in the present study were of high purity and the influence of minor impurities in the CPs on the formation of PCDD/F was not examined. It can not be excluded that specific impurities are in particular responsible for the observed formation of PCDD/F.

4. Conclusion

The in vitro studies confirm the suspicion that the biochemical formation of PCDD/F from precursors such as chlorophenols can take place in the human body and that this metabolic pathway may lead to a higher inner exposure to PCDD/F than up to now estimated by food analyses or duplicate studies. Thus, part of the observed higher excretion rates of higher chlorinated PCDD/F in relation to dietary intake found in the mass balances studies (Moser et al., 1996; Schrey et al., 1998) might be explained by peroxidase-catalyzed metabolic transformations of chlorophenols.

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Toxicology Letters 106 (1999) 201–208

Toxicology Letters

Methyl methanesulfonate and hydrogen peroxide differentially regulate p53 accumulation in hepatoblastoma cells

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Received 5 October 1998; received in revised form 12 March 1999; accepted 18 March 1999

Abstract

Genotoxic chemicals not only damage cellular DNA, but may also induce cell apoptosis if they are lethal to the cell. p53, Bcl-2 and Bax play important roles in the regulation of genotoxic chemical induced cell apoptosis. Since the mechanisms by which cellular DNA damaged by different DNA-damaging chemicals may not be the same, we studied the involvement of p53, Bcl-2 and Bax in apoptosis induced by methyl methanesulfonate (MMS) and hydrogen peroxide (H_2O_2). H_2O_2 damages DNA by free radical generation and MMS damages DNA by DNA methylation. At non-lethal doses, both H_2O_2 and MMS induced high level of p53 protein accumulation. Nevertheless, while the amount of p53 protein increased with the dose of MMS and the occurrence of apoptotic cell death events, H_2O_2 doses that induce cell apoptosis attenuated the p53 protein accumulation level. Lethal MMS treatment also increased Bax, but not Bcl-2 expression, whereas in H_2O_2 induced apoptosis, the level of both Bcl-2 and Bax declined. These results indicate that toxic chemicals differentially regulate the accumulation of p53 protein. Thus, the pathways of toxic chemicals induced cell apoptosis are different and independent. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Apoptosis; MMS; H_2O_2; p53; Bcl-2; Bax

1. Introduction

Many chemical compounds are toxic. Cells attacked by genotoxic chemicals result in genomic DNA damage. Destructive DNA damage may induce cell apoptosis (Lowe et al., 1993; Midgley et al., 1995), and this eliminates those cells whose DNA are not repairable and helps to prevent the cells from neoplastic transformation. The mechanisms by which genotoxic chemicals damage cells may be different; some by free radical production,

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PII: S0378-4274(99)00067-3

such as hydrogen peroxide (H_2O_2) (Kappus, 1987), and some by DNA alkylation, such as methyl methanesulfonate (MMS) (Beranek, 1990). H_2O_2 produces the hydroxyl radical ($HO^{\bullet}$) and generates a prooxidant status in the cell, while MMS can react directly and introduce the methyl group into DNA (Beranek, 1990).

The tumor suppressor p53 is a nuclear phosphoprotein involved in cell cycle control (Diller et al., 1990; Kastan et al., 1991; Kuerbitz et al., 1992), suppression of cell transformation (Michalovitz et al., 1990; Donehower et al., 1992), and apoptotic cell death (Clarke et al., 1993; Lowe et al., 1993). Cells exposed to ultraviolet light (UV) or γ-radiation rapidly increases their p53 level through a post-translational mechanism (Maltzman and Czyzyk, 1984; Kastan et al., 1991; Kuerbitz et al., 1992). Accumulation of p53 protein within the nucleus of DNA-damaged cells arrest the cell cycle at the G1 and S phase boundary (Kastan et al., 1991; Kuerbitz et al., 1992), and this is thought to allow cells to repair their damaged DNA before DNA synthesis initiation (Smith et al., 1996). Unsuccessful in DNA repair may result in the introduction of point mutation or deletion in the cell genome (Maltzman and Czyzyk, 1984; Kastan et al., 1991).

Besides p53, the Bcl-2 family is also important in the regulation of cell apoptosis. Bcl-2 was first identified as a novel transcript associated with the t(14;18) chromosomal translocation which occurs in most follicular lymphoma (Bakhshi et al., 1985; Tsujimoto et al., 1985; Cleary et al., 1986; Tsujimoto and Croce, 1986). Bcl-2 has been localized to mitochondria, endoplasmic reticulum and nuclear membranes (Krajewski et al., 1993). Cell death suppression by Bcl-2 have been characterized by various cell types. Bcl-2 inhibits cell apoptosis induced by free radicals (Krajewski et al., 1993), chemotherapeutic drugs (Miyashita and Reed, 1992; Hsu et al., 1994) and tumor necrosis factor (Fernandez et al., 1994; Talley et al., 1995). Another Bcl-2 family, Bax (Bcl-2 associated X protein), functions to promote cell death. Bax can form heterodimer with Bcl-2, the ratio of Bcl-2/Bax determines the survival or death of cells following an apoptotic stimulus (Oltvai et al., 1993). The expression of Bax can be regulated by p53 at the transcription level (Miyashita and Reed, 1995). Genotoxic agents induced cell apoptosis is also involved the expression of Bax (Zhan et al., 1994).

We report here that the expression of p53, Bcl-2 and Bax are differently regulated in apoptosis induced by different genotoxic chemicals. Treatment of cells with H_2O_2 or MMS stimulated p53 accumulation effectively at non-lethal doses. However, while the level of p53 increased in a dose-dependent fashion in cells treated with lethal doses of MMS, H_2O_2 at doses that induced cell apoptosis decreased p53 protein accumulation levels significantly. Lethal MMS treatment also increased Bax expression whereas lethal H_2O_2 treatment decreased Bcl-2 expression.

2. Materials and methods

2.1. Chemicals and antibodies

DMEM medium, L-glutamine, penicillin, streptomycin, and fetal calf serum were purchased from GIBCO/BRL (Gaithersburg, MD, USA). The culture dishes (Falcon) were purchased from Baxter (McGaw Park, IL, USA). MMS was purchased from Aldrich (Milwaukee, WI, USA). H_2O_2 was purchased from Merk (Darmstadt, Germany). Anti-p53 (Ab-2) antibody was purchased from Oncogene Science (Uniondale, NY, USA). Anti-Bcl-2 antibody, protease K and RNase A were purchased from Boehringer Mannheim (Mannheim, Germany). Anti-Bax antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-α-tubulin antibody was purchased from Zymed (Carlton Court, CA, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and genotoxic chemicals treatment

Hep G2 human hepatoblastoma cells were maintained in DMEM medium containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in an incubator containing 5% CO_2 at 37°C. MMS was freshly prepared in

DMSO and H_2O_2 was diluted in H_2O before being added to the culture dishes. The viable and dead cells were determined by chromosomal condensation, DNA fragmentation, and trypan blue exclusion assays and the percentage of dead cells were calculated as the ratio of dye-permeable cells to the total cell number.

2.3. Western blotting

The cells were washed with phosphate buffered saline (PBS) and lysed in ice cold RIPA buffer (25 mM Tris–HCl pH 7.2, 0.1% SDS, 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aporotinin, and 5 μg/ml leupeptin). Protein concentrations were determined by a BCA protein assay kit (Pierce., Rockford, IL, USA). A sample of 50 μg of each protein were loaded onto SDS-polyacrylamide gel. After electrophoresis, the protein was transferred to Hybond PVDF membrane (Amersham, Buckinghamshire, UK). The PVDF was blocked with blocking solution (20 mM Tris–HCl pH 7.6, 137 mM NaCl, 0.1% BSA) for 1 h and then reacted with antibody for 1 h. The membrane was then washed with blocking solution without BSA, and incubated with horseradish peroxidase-linked secondary antibody for 1 h, and after washing again, the levels of protein were analyzed by enhanced chemiluminescence with an ECL Western blotting detection system (Amersham, Arlingtn Height, IL, USA). After the performance of enhanced chemiluminescence reactions, total protein on the membrane were stained with Coomassie brilliant blue R250 solution to confirm equal loading of each sample.

2.4. DNA fragmentation analysis

Cells 48 h post-chemical treatment were harvested, washed with PBS and incubated in lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 0.5% sarkosyl, 1mg/ml protease K) at 50°C for 5 h. RNase A (final concentration 1 μg/ml) was added into the lysis buffer later. After an additional 1 h incubation at 50°C, the DNA were extracted with phenol–chloroform–isoamyl alcohol (25:24:1), and the DNA fragment were analyzed by agarose gel electrophoresis.

2.5. Acridine orange staining assay

The cells were harvested after treatment with MMS for 12 h or after treatment with H2O2 for 24 h. Cell suspension (5 μl) was mixed on a slide with an equal volume of acridine orange solution (10 μg/ml in PBS). Green fluorescence was detected between 500 and 525 nm using a microscope.

3. Results

3.1. Effects of non-lethal and lethal doses of MMS treatment on p53 accumulation

We treated the cells with various doses of H_2O_2 and MMS and assayed the level of p53 expression in response to the onset of apoptosis. Both the non-lethal MMS dose (200 μM) and lethal MMS dose (600 μM) treatment stimulated cellular p53 protein accumulation, and while the non-lethal MMS treatment only induced a low p53 accumulation level, the lethal MMS treatment stimulated a high p53 protein accumulation level. At 8 h post-MMS treatment, 600 μM MMS induced about a 30-fold p53 protein accumulation level compared to the 200 μM MMS treatment (Fig. 1A). The 600 μM MMS treatment induced chromosomal condensation and an apoptotic DNA ladder, and the 200 μM MMS treatment did not induce chromosomal condensation and an apoptotic DNA ladder of the cells (Figs. 3 and 4). In the dose–response assay, increased MMS doses resulted in an increase in both the p53 accumulation level and the dead cell percentage in a dose-dependent manner (Fig. 1B). The percentages of apoptotic cells after 72 h were 0.5, 0.5, 0.6, 27, and 82% for treatment with 50, 100, 200, 300, and 500 μM MMS, respectively.

H_2O_2 treatment yielded totally different results. Both the non-lethal (150 μM) and lethal (800 μM) H_2O_2 treatments stimulated cellular p53 protein accumulation rapidly within 4 h, but with the lethal dose (e.g. 800 μM) the cells accumulated a lower p53 level compared with that in cells treated

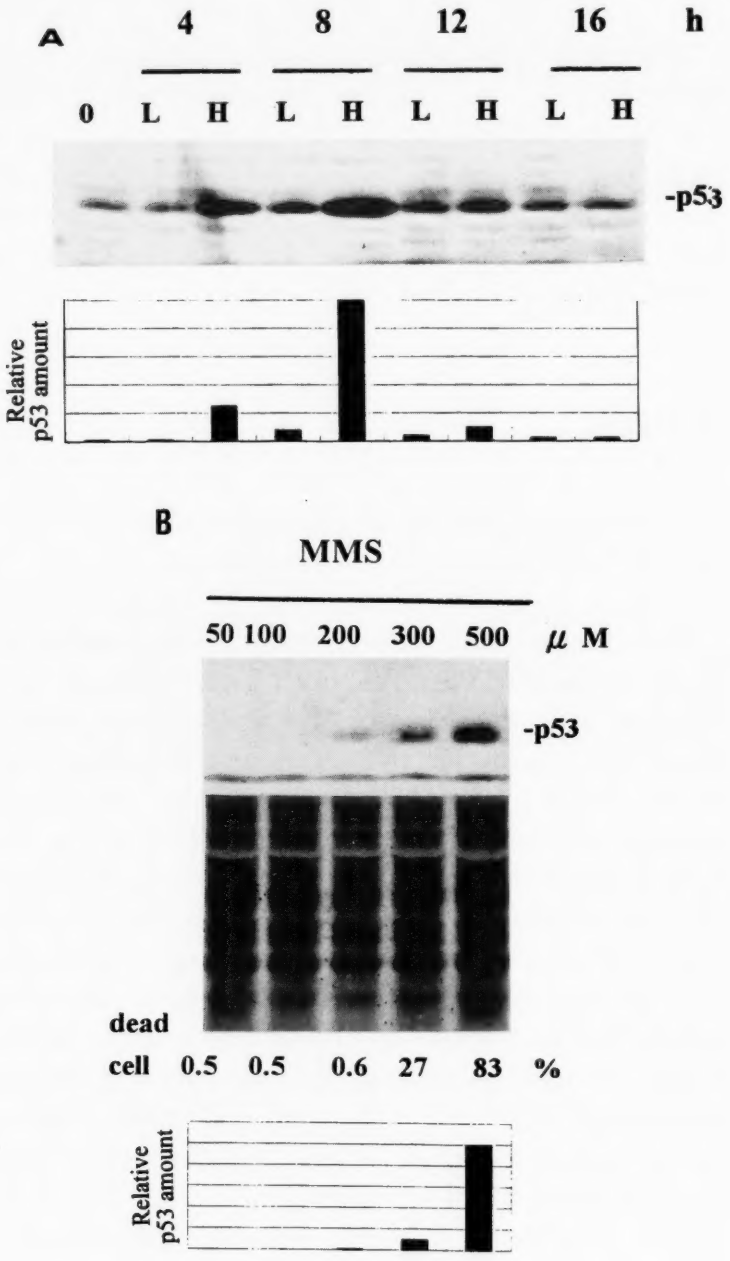

Fig. 1. Effect of MMS doses on p53 protein accumulation and cell viability. Western blotting of protein (50 μg) extracted from Hep G2 cells treated with: panel A, 200 μM MMS (L) or 600 μM MMS (H) for different times as indicated, or panel B, various doses of MMS as indicated for 8 h, were analyzed to determine the p53 protein level by using p53 monoclonal antibody. In panel B, after the ECL reactions were performed, the membranes were stained with Coomassie blue to prove equal loading of each sample. The percentages of dead cells after 72 h are given below.

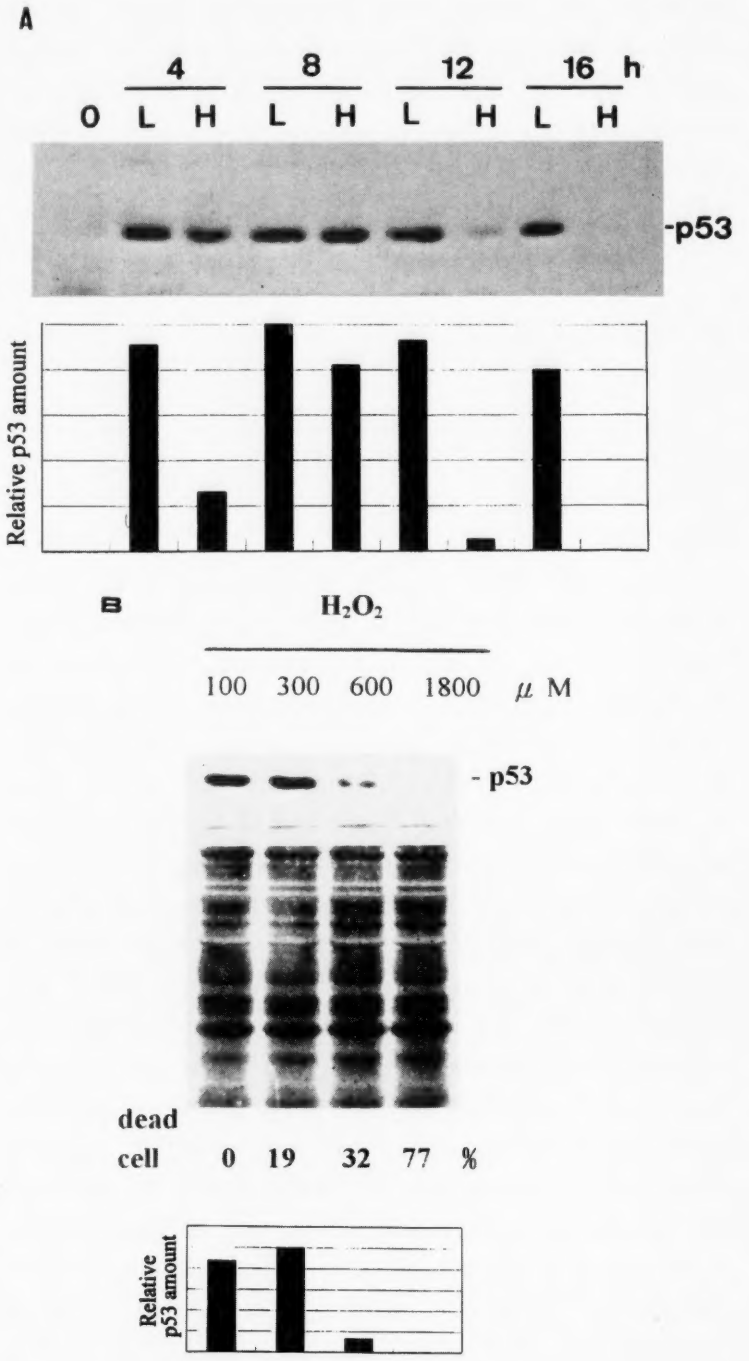

Fig. 2. Effect of H_2O_2 doses on p53 protein accumulation and cell viability. Western blotting of protein (50 μg) extracted from Hep G2 cells treated with: panel A, 150 μM H_2O_2 (L) or 800 μM H_2O_2 (H) for different times as indicated, or panel B, various doses of H_2O_2 as indicated for 12 h, were analyzed to determine the p53 protein level by using p53 monoclonal antibody. In panel B, after the ECL reactions were performed, the membranes were stained with Coomassie blue to prove equal loading of each sample. The percentages of dead cells after 72 h are given below.

with the non-lethal dose (e.g. 150 μM) of H_2O_2 (Fig. 2A). Treatment with H_2O_2 at doses that are capable of inducing cell apoptosis (i.e. 600 μM

and higher) resulted in a decrease in the level of p53 accumulation in cells and an increase in the percentage of apoptotic cells in a dose-dependent manner (Fig. 2B). The percentages of apoptotic cells after 72 h were 0, 19, 32, and 77% for treatment with 100, 300, 600, and 1800 μM H_2O_2, respectively. H_2O_2 induced cell death is apoptotic and the 800 μM H_2O_2 treatment induced chromosomal condensation and an apoptotic DNA ladder, while the 150 μM H_2O_2 treatment did not induce chromosomal condensation and an apoptotic DNA ladder (Figs. 3 and 4).

3.3. Effects of non-lethal and lethal doses of MMS and H_2O_2 treatment on Bcl-2 and Bax expression

The expression of Bcl-2 and Bax is correlated to genotoxic chemical induced cell apoptosis, and the transcription of Bax is directly regulated by p53. Therefore, we assayed the expression of Bcl-2 and Bax in non-lethal and lethal MMS or H_2O_2 treated cells. The cells were treated with various doses (from non-lethal to lethal) of MMS or H_2O_2. Increased doses of MMS treatment did not change the expression level of Bcl-2, even at the lethal doses. However, increased H_2O_2 doses decreased Bcl-2 expression in a dose-dependent manner. Increased MMS doses resulted in the

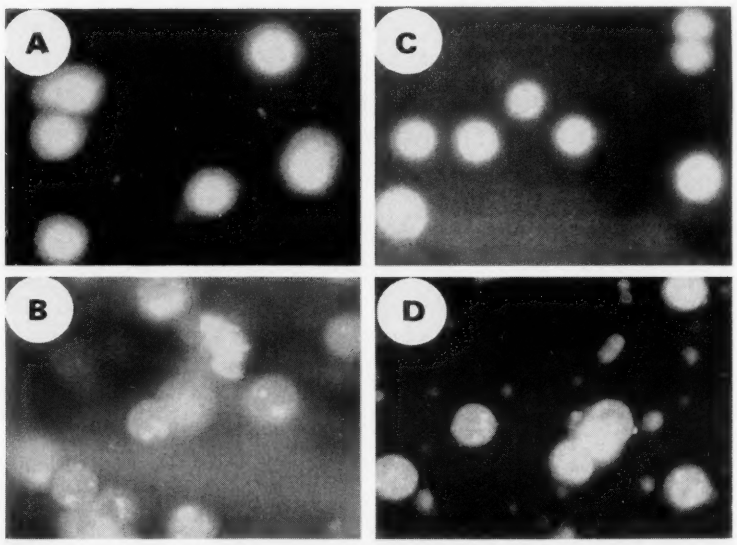

Fig. 3. Chromosomal condensation of Hep G2 cells treated with (A) 200 μM MMS for 12 h, (B) 600 μM MMS for 12 h, (C) 150 μM H_2O_2 for 24 h, and (D) 800 μM H_2O_2 for 24 h.

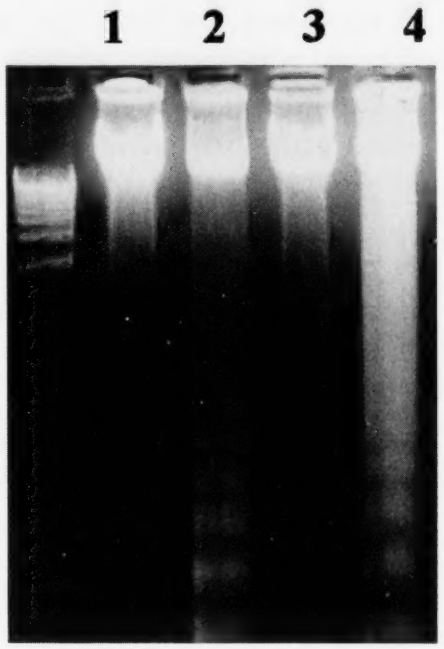

Fig. 4. DNA fragmentation induced by lethal or non-lethal MMS and H_2O_2 treated cells. Gel electrophoresis of DNA extracted from Hep G2 cells treated with 150 μM H_2O_2 (lane 1), 800 μM H_2O_2 (lane 2), 200 μM MMS (lane 3) or 600 μM MMS (lane 4) for 48 h.

increased expression level of Bax but increased H_2O_2 doses resulted in a decline in Bax level (Fig. 5). The expression level of Bax in MMS and H_2O_2 treated cells correlated well with the accumulation level of p53 protein induced by MMS and H_2O_2.

4. Discussion

The genotoxic chemicals induced cell apoptosis are determined by the mechanisms by which the genotoxic chemicals damaged the cells. Disparate genotoxic chemical with disparate mechanism of action to induce cell death suggests that the onset of cell death can be determined by the relative expression levels of cellular apoptosis promotion or suppression genes. The cellular response induced by genotoxic chemical is dependent on both the nature and the concentration of the chemical. MMS, γ-irradiation, and UV irradiation were reported to differentially regulate the activation of Jun kinase (JNK) and the non-receptor tyrosine kinase c-abl in murine fibroblasts (Liu et al., 1996). It is generally agreed that the activities of cellular signal transduction factors control the

expression of their target genes. The differential regulation of signal transduction pathways by different genotoxic agents indicate that the cellular apoptosis related genes can be differentially regulated in apoptosis induced by different genotoxic chemicals.

The rapid accumulation of p53 is well known in cell response to genotoxic chemicals induced DNA damage or apoptosis. Both H_2O_2 and MMS are capable of inducing p53 protein accumulation at non-lethal doses. The accumulation of p53 protein in non-lethal doses of MMS and H_2O_2 treated cells probably served to repair the damaged DNA. In lethally MMS treated cells, both the cell death percentage and the p53 protein accumulation level increased in proportion to the MMS doses. However, increased H_2O_2 doses resulted in increased cell death numbers with a corresponding decrease in cellular p53 protein accumulation levels. There are many reports

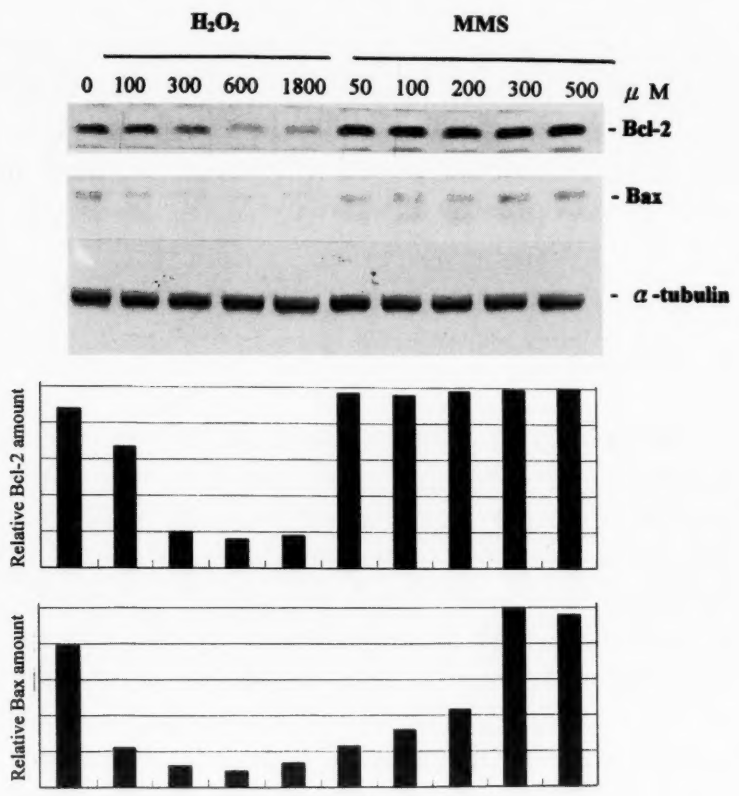

Fig. 5. Effect of H_2O_2 and MMS doses on Bcl-2 and Bax expression. Western blotting of protein (50 μg) extracted from Hep G2 cells treated with various doses of H_2O_2 or MMS as indicated for 16 h, were analyzed to determine the Bcl-2 (first panel) and Bax (second panel) level. The level of α-tubulin protein (third panel) were determined to prove equal loading of each sample.

demonstrating that in some cases, dysfunction of p53 or its target protein sensitizes cells to apoptosis (Fan et al., 1995; Hawkins et al., 1996; Wahl et al., 1996; Fan et al., 1997). Thus, it seems that p53 protein accumulation ability can be differentially regulated in apoptosis induced by different genotoxic chemicals.

Cellular post-translational modification mechanisms regulate the accumulation of p53 protein. The alkylating agent MMS can react directly with DNA, whereas the toxic effects of H_2O_2 are dependent on hydroxyl radical ($HO^{\bullet}$) formation via the Harber–Weiss reaction (Haber and Weiss, 1934):

$$H_2O_2 + O_2^- + H^+ \rightarrow HO^{\bullet} + O_2 + H_2O$$

or the Fenton reaction (Fenton, 1894):

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow HO^{\bullet} + Fe^{3+} + H_2O$$

The hydroxyl radical is highly reactive and can damage many cellular compounds, meanwhile, these radicals may also attack other cellular protein containing free SH-groups that may affect the accumulation ability of p53 protein. In addition, besides attacking DNA via free radical generation, H_2O_2 can induce oxidative stress inside the cell and damage many other cellular components, such as cellular membranes, mitochondrial membranes, and endoplasmic reticulum by lipid peroxidation (Kappus, 1987). Therefore, both cytotoxic and genotoxic effects may exist in lethal H_2O_2 treated cells. The decreased p53 expression with lethal doses of H_2O_2 treatment may also result from the cytotoxic effect of H_2O_2 which make the cells incapable of accumulating p53 protein.

Lethal MMS treatment did not affect the expression of Bcl-2 but it did stimulate the expression of Bax, while lethal H_2O_2 treatment down-regulated the expression of both Bcl-2 and Bax. The increase in Bax in lethally MMS treated cells may result from the increased p53 level induced by lethal MMS treatment, and the decrease in Bax in lethally H_2O_2 treated cells may resulted from the decreased p53 level induced by lethal H_2O_2 treatment. The increase of apoptosis promotion protein p53 and Bax may play an important role in apoptosis triggered by MMS, and the

decline in apoptosis suppression protein Bcl-2 level may be important in H_2O_2 induced cell apoptosis.

In conclusion, distinct genotoxic chemicals damage cells with distinct mechanisms. Besides a differentially activate cellular stress response signal transduction factor, different genotoxic chemicals can differentially regulate apoptosis-related genes. These results indicate that genotoxic chemicals induce cell apoptosis by different and independent pathways.

Acknowledgements

This work was supported by grant from the National Science Council, NSC 87-2314-B002-036.

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Toxicology Letters 106 (1999) 209–214

Toxicology Letters

Protein oxidation in thyroid hormone-induced liver oxidative stress: relation to lipid peroxidation

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Received 30 November 1998; received in revised form 18 March 1999; accepted 19 March 1999

Abstract

The influence of thyroid hormone administration (daily doses of 0.1 mg of 3,3′,5-triiodothyranine (T_3)/kg for 1–3 consecutive days) on rat liver protein oxidation was investigated in relation to the calorigenic and lipid peroxidative actions of the hormone. T_3 treatment elicited a progressive enhancement in the serum levels of the hormone, the rectal temperature of the animals, and in the rate of O_2 uptake of the liver, changes that are significantly correlated and evidence the development of thyroid calorigenesis. Liver lipid peroxidation was augmented by T_3 administration as determined by the tissue content of thiobarbituric acid reactants, with a maximal effect (3.1-fold increase) being found at 2 days after treatment, whereas protein oxidation measured by the content of protein hydrazone derivatives exhibited a maximal 88% increase at 3 days. Maximal rates of lipid peroxidation occur at 1 day after the administration of T_3, whereas those of protein oxidation are attained after treatment with three daily doses of T_3, time at which the former levels off. It is concluded that T_3 administration induces a substantial enhancement in hepatic protein oxidation, in addition to lipid peroxidation, that seems to be due to the higher oxidative stress status conditioned in the liver by thyroid calorigenesis. Both processes exhibit a differential time course of changes, that may represent differences in the susceptibility of target molecules to free radical attack and/or in the efficiency of repair mechamisms. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Thyroid hormone; Oxidative stress; Protein oxidation; Lipid peroxidation; Rat liver

1. Introduction

Cellular oxidative stress represents an imbalance between the steady-state production of reactive oxygen (ROS) and nitrogen (RNS) species and the rate of their consumption by antioxidants

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PII: S0378-4274(99)00068-5

in favor of the former, that may result in pathophysiological events (Sies, 1993). The extent of the oxidative stress status of tissues correlates with the specific metabolic rate of individuals of different mammalian species (Cutler, 1985), and, thus, the acceleration of the aerobic metabolism in a given animal species exacerbates oxidative stress, as reported for thyroid hormone-induced calorigenesis in experimental animals (Fernández and Videla, 1989) and man (Videla et al., 1988; Wilson et al., 1989). In this condition, the signal transduction pathway involves thyroid hormone binding to specific nuclear receptors in target cells, leading to the expression of enzymes related to redox processes and enhancement in the total rate of O_2 consumption (Oppenheimer et al., 1996). In the liver, thyroid hormone-induced O_2 uptake involves higher rates of superoxide radical ($O_2^{\cdot -}$) and/or hydrogen peroxide (H_2O_2) generation in microsomal, mitochondrial, and peroxisomal fractions (Fernández et al., 1985; Fernández and Videla, 1993a), as well as that of cytosolic nitric oxide ($^{\cdot}NO$) (Fernández et al., 1997). In addition, hyperthyroidism enhances Kupffer cell functioning, thus representing an alternate source of free radical species to that induced in parenchymal cells (Tapia et al., 1997). Therefore, oxidative stress is established in the liver by the enhanced free radical activity induced by thyroid calorigenesis coupled with derangement in antioxidant mechanisms, namely, depression in the activity of superoxide dismutase and catalase (Fernández et al., 1988) and depletion of glutathione (GSH) (Fernández et al., 1988; Morini et al., 1991), α-tocopherol, β-carotene, and lycopene (Giavarotti et al., 1998). This pro-oxidant state induced in the liver by hyperthyroidism may favor the oxidative deterioration of susceptible biomolecules with the consequent alteration or loss of their functions, as shown by the enhancement in the peroxidation of polyunsaturated fatty acids of membrane phospholipids (Fernández et al., 1985; Fernández and Videla, 1989; Morini et al., 1991). Increased liver lipid peroxidation by hyperthyroidism is paralleled by a substantial increase in the sinusoidal efflux of lactate dehydrogenase assessed in perfusion experiments, suggesting a derangement in the permeability properties of the plasma membrane of liver cells secondary to the augmented free radical activity imposed by thyroid calorigenesis (Fernández et al., 1991).

The ability of free radicals to damage biological molecules is not restricted to lipids, as these species can also interact with a number of biomolecules including proteins (Stadtman, 1990). The free radical-protein interaction has been demonstrated in in vitro conditions in which free radicals are generated by the controlled thermal decomposition of azo compounds (Lissi et al., 1991), radiolysis, or by metal ion-catalyzed reactions (Stadtman, 1990). In view of these considerations, the aim of the current work is to investigate the influence of thyroid hormone-induced liver oxidative stress on hepatic protein oxidation, as a possible factor altering protein metabolism and function in this hormonal dysfunction (Loeb, 1996). Results obtained are correlated with changes in hepatic lipid peroxidation, assessed by the thiobarbituric acid index.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats weighing 200–250 g were fed ad libitum and maintained on a 12 h light/dark cycle. The animals received daily i.p. injections of either L-3,3′,5-triiodothyronine (T_3) (0.1 mg/kg) or equivalent volumes of T_3 diluent (0.1 N NaOH) (controls) for 1–3 consecutive days, and studies were performed 24 h after the last treatment. At each experimental time studied, serum T_3 levels were measured by a Gamma Coat [^{125}I]T_3 radioimmunoassay kit (Baxter Health-Care, Cambridge, MA), the rectal temperature of the animals was determined with a thermocouple (Model 8112-20, Cole-Parmer Instrument, Chicago, IL), and the rate of O_2 consumption of the liver was assessed polarographically in perfusion experiments as described previously (Fernández et al., 1991). All animals employed received humane care according to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health Publication 86–23).

2.2. Biochemical assays

Rats were anesthetized with sodium pentobarbital (50 mg/kg ip) and the livers were perfused in situ with 200 ml of a cold solution containing 140 mM KCl and 10 mM potassium phosphate buffer, pH 7.4, to remove blood. Liver protein oxidation was assayed by the reaction of 2,4-dinitrophenylhydrazine with protein carbonyls according to Reznick and Packer (1994), lipid peroxidation was determined by the thiobarbituric acid assay (TBARS) as described by Buege and Aust (1987), and the protein content of liver homogenates was measured according to Lowry et al. (1951).

All reagents used were obtained from Sigma Chemical (St. Louis, MO). Results shown correspond to the means ± S.E.M. for the number of separate experiments indicated. One-way ANOVA and the Newman–Keuls' test assessed the statistical significance of differences between mean values.

3. Results and discussion

The administration of T_3 to fed rats elicited a progressive enhancement in the serum levels of the hormone (Fig. 1A), with concomitant increases in the rectal temperature of the animals (Fig. 1B) and in the rate of O_2 consumption of the liver (Fig. 1C). These changes induced by T_3 are significantly correlated [serum T_3 levels versus rectal temperature ($r = 0.86$, $P < 0.01$) and liver O_2 uptake ($r = 0.98$, $P < 0.001$); rectal temperature versus liver O_2 uptake ($r = 0.93$, $P < 0.001$)] and evidence the development of a calorigenic response. T_3-induced acceleration of hepatic respiration (Fig. 1C) involves a component suppressed by the antioxidants desferrioxamine and allopurinol, comprising 16–25% of the net increase in hepatic O_2 uptake that seems to represent O_2 equivalents used in free radical-dependent processes (Fernández and Videla, 1993b). Lipid peroxidation, as an O_2-dependent free radical-mediated process, is also progressively augmented upon T_3 administration as measured by the hepatic content of TBARS, which exhibits a maximal effect (3.1-fold increase) at 2 days after treatment (Fig. 2). This finding is in agreement with earlier reports measuring TBARS production in liver homogenates (Fernández et al., 1985) or the biliary release of TBARS in the anesthetized rat (Fernández and Videla, 1996), chemiluminescence by rat liver homogenates (Fernández et al., 1985) and rabbit liver mitochondria (Marzoev et al., 1982), light emission of the in situ rat liver (Fernández et al., 1988), and microsomal hydroperoxide formation (Landriscina et al., 1988; Morini et al., 1991). In man, thyroid hormone-induced prooxidant condition (Wilson et al., 1989) is associated with elevated circulating and urinary lipid peroxidation indicators, which are normalized by propylthiouracil treatment (Videla et al., 1988; Lissi et al., 1992a).

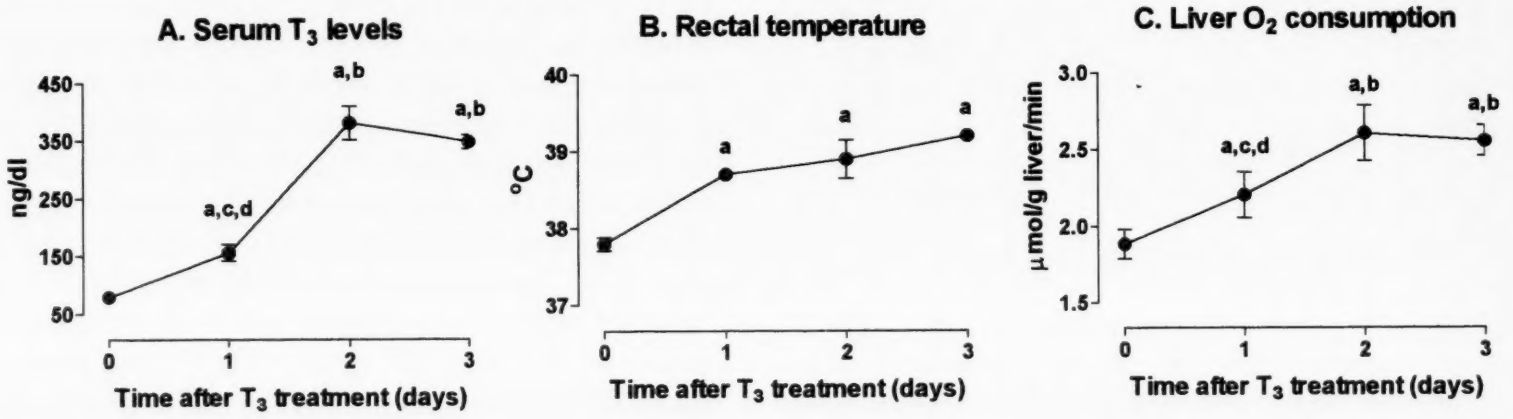

Fig. 1. Serum T_3 levels (A), rectal temperature of the animals (B), and liver O_2 consumption (C) in control rats and T_3-treated animals. Rats were given daily doses of 0.1 mg T_3/kg for one to three consecutive days or T_3 vehicle (controls, time zero). Values shown represent means ± S.E.M. for 4–9 animals per experimental group. Significance studies: $P < 0.05$ versus controls (a), T_3-treated 1 day (b), T_3-treated 2 days (c), and T_3-treated 3 days (d).

In addition to lipid peroxidation, hyperthyroidism enhances hepatic protein oxidation, as evidenced by the significant 88% increase in the content of protein hydrazone derivatives at 3 days after T_3 treatment (Fig. 2). This effect may be due to the increased generation of ROS induced by T_3 (Fernández et al., 1985; Fernández and Videla, 1993a), leading to the formation of carbonyl derivatives mainly occurring in arginyl, prolyl, lysyl, and histidyl residues in proteins (Stadtman, 1990; Reznick and Packer, 1994). Also, T_3-induced ROS formation can promote the conversion of cysteinyl residues to protein-protein disulfide conjugates or to mixed-disulfide derivatives (Stadtman, 1990), whereas T_3-induced NO generation (Fernández et al., 1997) may lead to protein oxidation and/or nitration through peroxynitrite formation (Radi et al., 1995). The biological significance of the oxidative modification of proteins in T_3-induced liver oxidative stress can be visualized at two levels, namely: (i) loss of protein function and (ii) increased protein degradation. Under high rates of ROS and RNS input, the oxidative modification of enzymes can occur with the consequent reduction in enzyme activity (Stadtman, 1990; Lissi et al., 1992b). The inactivation of hepatic antioxidant enzymes has been described in several in vivo conditions involving oxidative stress in the tissue including hyperthyroidism (Lissi et al., 1992b), that determines a decrease in the activity of superoxide dismutase and catalase (Fernández et al., 1988) and in the content of cytochrome P450 (Fernández et al., 1985). In agreement with this contention, inactivation of superoxide dismutase by H_2O_2 (Bray et al., 1974) and of catalase by $O_2^{\cdot -}$ (Kono and Fridovich, 1982) has been reported in in vitro conditions. In addition to enzyme inactivation, thyrotoxicosis in mammals results in stimulation of both synthesis and degradation of protein, with predominance of degradation, as evidenced by the increase in protein catabolism, negative nitrogen balance, and loss of protein from muscle and other body stores (Loeb, 1996). It is conceivable that T_3-induced liver protein oxidation (Fig. 2) and degradation may be associated, as the oxidative modification of proteins by oxidative stress renders them highly susceptible to proteolytic attack by most proteases and/or by the multicatalytic proteinase (Rivett, 1985; Stadtman, 1990). It is concluded that thyroid hormone-induced liver oxidative stress leads to a substantial enhancement in lipid and protein oxidation, effects that correlate with a derangement in associated functions, namely, membrane permeability (Fernández et al., 1991) as well as protein turnover (Loeb, 1996) and enzyme inactivation (Fernández et al., 1985, 1988). The maximal changes induced by T_3 widely differ in magnitude, as evidenced by the 3.1-fold increase in hepatic lipid peroxidation compared to the 88% enhancement in protein oxidation (Fig. 2). Furthermore, the calculated rates of protein oxidation and lipid peroxidation induced by consecutive thyroid hormone administration exhibit differential time course of changes. In fact, the maximal rate of lipid peroxidation (0.18 nmol/mg protein/day; from Fig. 2) occurs at 1 day after the administration of a single dose of thyroid hormone, whereas that of protein oxidation (0.67 nmol/mg protein/day; from Fig. 2) is attained after the administration of three daily doses of T_3, time at which the former process levels off. These changes could be due to possible differences in the susceptibility of polyunsaturated fatty acids and proteins to free radical attack and/or in the efficiency of the repair or replacement systems that may be triggered in this condi-

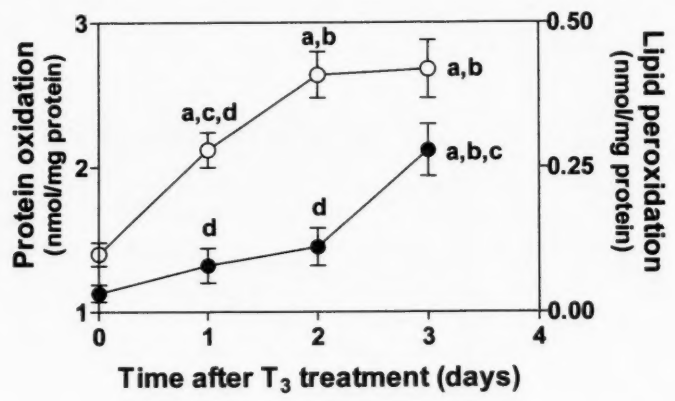

Fig. 2. Time course of the effect of T_3 treatment on liver protein oxidation (POX) (●) and lipid peroxidation (LP) (○)in the rat. Animals were given daily doses of 0.1 mg T_3/kg for 1–3 consecutive days or T_3 vehicle (controls, time zero). Values shown represent means (S.E.M. for 4–9 animals per experimental group. Significance studies: $P < 0.05$ versus controls (a), T_3-treated 1 day (b), T_3-treated 2 days (c), and T_3-treated 3 days (d).

tion, however, additional studies are needed to clarify this suggestion.

Acknowledgements

This work was supported by grant 1970300 from FONDECYT (Chile).

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Toxicology Letters 106 (1999) 215–228

Toxicology Letters

Na^+:H^+ exchange inhibition induces intracellular acidosis and differentially impairs cell growth and viability of human and rat hepatocarcinoma cells

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Received 8 July 1998; received in revised form 18 March 1999; accepted 23 March 1999

Abstract

Amiloride and its more potent analog, hexamethylene amiloride (HMA), inhibits Na^+:H^+ exchange and decreases intracellular pH in a concentration-dependent way in two human hepatocarcinoma cell lines and in a rat hepatocarcinoma cell line that differs in its phenotypic characteristics, resembling the clinical situation encountered in human hepatocarcinomas. After 24 h of exposure, DNA synthesis and cell protein content of the cultures decreases according to the concentration of the drugs and in parallel to Na^+ exchange inhibition and the drop in pH_i promoted. RNA and protein syntheses are less sensitive to its action. The above effects induced by HMA are accompanied by an abrupt decrease in cell viability and lysosomal integrity at 24 h. These effects develop gradually with the exposure time as does the increase in free radical production. Decreased viability is totally or partially restored by *N*-acetylcysteine or deferoxamine, but the degree of intracellular acidification produced is not. These results tend to suggest that intracellular acidification can diminish cell growth and provoke cytotoxic cell death by diminishing reduced glutathione (GSH) levels and impairing lysosomal integrity, reflecting the sensitivity of hepatocarcinoma cells to Na^+ exchange inhibition and intracellular acidosis. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hexamethylene amiloride; Human hepatocarcinoma; Intracellular pH; Na^+:H^+ exchange

1. Introduction

Human hepatocellular carcinoma (HCC) is one of the malignancies for which few effective treatment strategies actually exist, mainly due to the advanced stage of disease at the moment of diag-

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0378-4274/99/$ - see front matter © 1999 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0378-4274(99)00072-7

nosis and to the heterogeneous nature of HCC (Venook, 1994). Among the different therapeutic strategies, the main chemotherapeutic treatment requires intrahepatic arterial infusion of anticancer agents, to which the tumor appears refractive due to the intrinsic multidrug resistance frequently observed in hepatic malignancies and in hepatocarcinogenesis (Thorgeirsson et al., 1987), which diminishes the intracellular level of anticancer drugs required for its therapeutic action. Anthracyclines of different lipophilicity have been assayed in an attempt to augment its therapeutic action in the treatment of non-resectable liver tumors (Rivory et al., 1996). Another approach has been the infusion of a combination of several anticancer drugs during the surgical resection of the tumor mass, in the cases in which it is possible, with limited results due to the onset of undesirable toxic side-effects in the remaining, normally regenerating liver (Tamura et al., 1992).

A substantial difference observed in solid tumors with respect to the surrounding healthy tissue (Kallinowski and Vaupel, 1988) involves the interstitial pH, which is more acidic in tumors, although under most circumstances the intracellular pH (pH_i) of tumor cells remains more basic than that of the extracellular milieu (Gerweck and Seetharaman, 1996). The reasons for these differences are related to the poor vascularization of the tumor mass, the glycolytic rate of lactic acid formation and hypoxia (Harguindey, 1994; Stubbs et al., 1994). It has been proposed that these differences could be of value in the treatment of tumors (Wike-Hooley et al., 1984; Tannock and Rotin, 1989), perhaps as the basis for establishing a possible combination therapy (Harguindey et al., 1989; Newell and Tannock, 1989).

pH_i homeostasis seems to be directly involved in tumor cell growth and metastatic progression. Systemic acidosis, as well as the use of amiloride and its analogs, appear to inhibit DNA synthesis and tumor cell growth, both in vitro and in vivo in several animal and human tumors (Kallinowski and Vaupel, 1988). It has been observed that pH_i is directly and almost exclusively regulated by an active Na^+:H^+ exchange (NHE) system in a human derived liver tumor cell line (Strazzabosco et al., 1995), with amiloride acting as an inhibitor of DNA synthesis stimulated by fetal calf serum (FCS) and tumor growth factor α (TGF-α) at rather high concentrations. However, the response to intracellular acidification of multidrug resistance mechanisms, present in the majority of human hepatomas (Strazzabosco and Boyer, 1996), or its cytotoxic or cytostatic effects have not been studied yet in hepatomas, although regulation of pH_i has been well characterized in normal hepatocytes (Huang et al., 1992) and the effect of amiloride in modulating multidrug resistance transport in a rat hepatoma cell line has been documented by us (García-Cañero et al., 1998).

We were interested in studying the effects of the more potent amiloride derivative, hexamethylene amiloride (HMA), on pH_i maintenance in hepatocarcinoma cells in vitro in greater depth, as we had previously observed that subtoxic doses of amiloride and its most potent derivatives are capable of increasing the cytocidal effect of several anticancer drugs in an experimental hepatocarcinoma in tissue culture (Harguindey et al., 1995). Moreover, this effect was specific for tumor cells as compared with normal, immortalized hepatic cells. In this work, we study the effects of amiloride on growth, macromolecular synthesis, and going further, those of its derivative HMA on intracellular pH and intracellular organelle viability, in three hepatoma cell lines of human and rat origin, and differing phenotypes, in order to establish an in vitro liver tumor cell model. The purpose is to search for organulocellular target(s) as a possible mode of action of NHE inhibitors, based on promoting intracellular acidification, which may sensitize hepatomas to cell death and/or diminish tumor growth, and to overcome the negative effects of intrinsic and/or acquired chemoresistance encountered in the treatment of liver tumors.

The tumor cell lines we selected to study here differ in their metabolic characteristics and tumorigenicity. HepG2 cells, which are non-tumorigenic, have been found to behave more like normal, mature hepatocytes with regard to albumin synthesis, cytochrome P450-linked xenobiotic and bile acid metabolism, and hormone response (Knowles et al., 1980; Javitt, 1990), whereas cells from rat hepatocarcinoma in tissue culture

(HTC), derived from Wistar hepatocarcinoma 2188c, are devoid of many of these features (Thompson et al., 1966; Bassi et al., 1993). They have been characterized as a highly glycolytic, highly dedifferentiated hepatocarcinoma cell line (Schamhart et al., 1979; Cote and Chiu, 1987) with a depressed capacity for albumin secretion and impaired xenobiotic transforming metabolic pathways. HTC cells express mutated *p53* and lack of expression of the anti-apoptotic *bcl-2* gene (Evansstorms and Cidlowski, 1997). PLC PRF5 is a tumorigenic human cell line expressing part of the hepatitis B virus genome. In both cases, however, expression of several mutated oncogenes (*p53*, *Ha-ras* and c-*myc*) or α-fetoprotein secretion is detected, as in the case of clinically confirmed human hepatocarcinomas, whereas Hep G2 cells express wild-type *p53* and retinoblastoma protein (Jhiang et al., 1996), reflecting at the experimental level the behavior encountered in human liver cancer (Venook, 1994).

2. Materials and methods

2.1. Drugs and reagents

Amiloride was generously provided by Merck, Sharp and Dohme, Spain, as was the pure product, HMA, by Sigma-Spain. Cell-permeable fluorochromes BCECF, fluo-3 and calcein, all in their acetoxymethyl ester (AM) form, neutral red (NR) and rhodamine 123 (R123) were obtained from Molecular Probes, Inc. (Eugene, OR, USA) or Sigma-Spain. Culture media, FCS and antibiotics were from Imperial Laboratories, UK. All the other chemicals were of analytical grade. [*methyl*-^{3}H]Thymidine (specific activity 21 Ci/mmol), [5,6-^{3}H]uridine (specific activity 37.5 Ci/mmol) and L-[1-^{14}C]leucine (specific activity 50 mCi/mmol) were from New England Nuclear. Amiloride and HMA were dissolved in dimethyl sulfoxide (DMSO) at 100 mM and 20 mM, respectively, and stored in the dark at 4°C. For use, they were diluted in culture medium at the appropriate concentration to maintain DMSO below 0.1%. Control cultures received the same amount of DMSO alone.

2.2. Cell lines and culture conditions

Human hepatoblastoma, HepG2 (ATCC HB 8065) and human hepatocarcinoma PLC PRF5 (ECACC 85061113) were obtained from the American Type Culture Collection and the European Collection of Cell Cultures, respectively, and maintained in culture in 25-cm^2 tissue culture flasks (Nunc) at 37°C in a 5% CO_2:95% air atmosphere under sterile conditions, in a mixture of Ham's F-12–DMEM (1:1) medium and DMEM, respectively, in the presence of 10% FCS plus antibiotics, and subcultured weekly. HTC was kindly provided by Dr H.N. Christensen (Biological Chemistry Department, School of Medicine, University of Michigan at Ann Arbor, MI, USA) and cultured as above in DMEM medium in the presence of antibiotics and 5% FCS. For experimental manipulation, cells were seeded at 10,000 cells/cm^2 in the appropriate culture medium and cultured for 24 h before addition of drugs, fluorochromes or isotopically labeled metabolites. All the culture procedures were conducted under sterile conditions.

2.3. Determination of cell viability

Metabolic reduction of 3-(4,5)-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) was performed as described (Carmichael et al., 1987), except that the cultured cells, seeded onto 96-well microplates, were incubated for 120 min at 37°C before the completion of 4 or 24 h of incubation in the presence of amiloride or HMA or other agents. Blank wells received only 50 μl of phosphate-buffered saline (PBS). After solubilization of the formazan formed with 200 μl DMSO per well, optical density was recorded using a multiwell plate optical scanner (Labsystems Multiskan Bichromatic) at 540–690 nm. Results are expressed as a percentage of the values for control, untreated wells, done in quadruplicate, and are the mean ± S.E. of three different cultures.

2.4. Fluorimetric determination of organelle directed cytotoxicity

We used the retention of NR as a probe for lysosomal integrity, R123 retention to measure mitochondrial functionality, calcein retention as a probe for membrane permeability, and 2′,7′-dichlorofluorescin diacetate to detect free radical production using previously published methods (Danks et al., 1992) in cultures seeded onto 96-well microplates. The results were assessed using a fluorescent microplate reader (FLUOSTAR®, SLT, Austria) equipped with optical excitation and emission interference filters. Cells were incubated for 1 h at 37°C in the following fluorescent dyes: NR (100 μg/ml), R123 (10μg/ml), calcein-AM (10 μg/ml), or 2′,7′-dichlorofluorescin (100 μM), 1 h before the completion of HMA incubation. Treatment of loaded cells was as described, except for determination of mitochondrial R123 retention, in which cells were washed twice with DMEM and treated with 150 μM digitonin for 30 s at 37°C and washed again prior to fluorescence measurements to eliminate the fluorescence contribution from cytosolic R123 (Zoeteweij et al., 1994). In all cases, blanks were obtained from wells to which no fluorochrome was added. The following excitation–emission filters were used: NR (544–590 nm), R123 (485–538 nm), calcein (485–538 nm) and 2′,7′-dichlorofluorescin (485–538 nm). Results are expressed as percentage of control cultures, and are the mean ± S.E. of three separate cultures done n quadruplicate.

2.5. Determination of intracellular pH (pH_i), Na^+:H^+ exchange and Ca^{2+} (Ca_i^{2+})

Cultured cells seeded at 10,000 cells/cm^2 in 25-cm^2 culture flasks or 96-well plates were allowed to grow for 24 h, after which the medium was changed and loaded with 10 μM BCECF-AM or fluo-3-AM in the presence of 0.05% pluronic acid for 1 h at 37°C in culture medium as described (Danks et al., 1992; Jover et al., 1992), before HMA or amiloride treatment or after the 24-h treatment with HMA. They were then washed three times in Hanks' salt medium (pH 7.4). BCECF fluorescence was measured, at 32°C in a thermostat-controlled stirred cuvette, using using a Perkin Elmer LS5B fluorescence spectrofluorimeter set for a 432/500 excitation wavelength pair and 530 nm emission, after signal equilibration. To measure NHE activity, BCECF-loaded cells were acidified by a 20-mM potassium acetate pulse in the presence of 0–250 μM amiloride or 0–100 μM HMA, the slope of the pH_i recovery rate being a measure of the NHE activity. IC_{50} values were calculated from the inhibition curves obtained. The signal ratio was calibrated and converted to pH units as described elsewhere (James-Kracke, 1992). Intracellular fluo-3 fluorescence was measured on 96-well plates using the fluorescence microplate reader already described, set for an excitation–emission pair of 485–530 nm. Fluorescence calibration and Ca_i^{2+} conversion were done in each well using the ionomycin–Ca^{2+}–EGTA method (Jover et al., 1992). The results are expressed in terms of pH units and nM, respectively, and represent the mean ± S.E. of three separate experiments done in triplicate.

2.6. Determination of macromolecular synthesis and reduced glutathione (GSH) levels

Cells were seeded as described before onto 24-well plates. Parallel cultures were allowed to grow for 24 h in the presence of several amiloride concentrations up to 250 μM or HMA concentrations up to 100 μM, in triplicate, and 0.25 μCi/ml [^{3}H]thymidine, 0.25 μCi/ml [^{3}H]uridine or 0.1 μCi/ml L-[^{14}C]leucine. Afterwards, the culture medium was aspirated, quickly washed three times with cold PBS and three times with cold 0.1 N perchloric acid to eliminate soluble radioactivity. Radioactivity incorporated into the precipitate was solubilized with 0.1 N NaOH for 1 h at 37°C with agitation, and an aliquot was used to measure radioactivity by counting in a Wallach LS1410 liquid scintillation counter. Another aliquot was used to measure the protein content of the cultures (Bradford, 1976). Results, expressed as dpm incorporated per mg protein, were calculated as percentages of control, untreated cultures.

Reduced GSH levels in control and HMA-treated cultures were determined by the *o*-phtha-

laldehyde method of Hissin and Hilf (1976) and assessed in terms of the protein concentrations of the cultures. Values are the mean ± S.E. of three different cultures. As in all the above measurements, values were considered significantly different at $P < 0.05$ by the unpaired Student's t-test.

3. Results

Exposure to amiloride or HMA decreased NHE activity in the three tumor cell lines, as indicated in Table 1 and Fig. 1A–C, respectively, HMA being about six times more potent than amiloride in inhibiting NHE activity. At 24 h of exposure, 100 μM HMA decreased pH_i by about 0.7 pH_i units with respect to untreated cultures, as shown in Fig. 1 D–F. Amiloride promoted a decrease of only 0.2–0.3 pH_i units at the highest concentration employed (250 μM; results not shown). In an extended range of concentrations during the same time period, amiloride decreased macromolecular synthesis and cell content in the order DNA synthesis > RNA synthesis ≈ protein synthesis ≥ cell content in HTC cells, DNA synthesis ~ cell content > protein synthesis > RNA synthesis in HepG2 cells and protein synthesis ≈ cell content > DNA synthesis > > RNA synthesis in PLC PRF5 cells. HMA effects on the same cell lines were as follows: DNA synthesis ≈ cell content > RNA synthesis > protein synthesis in HTC, DNA synthesis = RNA synthesis ~ cell content > protein synthesis in HepG2 cells and DNA synthesis ≥ RNA synthesis > cell content > protein synthesis in PLC PRF5 cells, as shown in Table 2. The apparent discrepancies encountered between the action of HMA and that of amiloride could be a reflection of their different inhibitory properties, as amiloride, a Na^+ channel inhibitor, is a less specific NHE activity inhibitor than HMA. Moreover, the effects of HMA on the tumor cell lines could reflect differences in pH requirements of the mechanisms involved in macromolecular synthesis, or the different cellular energetic requirements needed. These may have been altered by exposure to inhibitors, as in all the three cell types, pH_i decreased as the HMA concentration increased. This change occurred more abruptly in hepatocarcinoma cells than in hepatoblastoma cells, and the minimum pH_i values differed, a finding that could be in accordance with differences in the pH_i set point for NHE activity in each case.

Table 1
IC_{50} (μM) of NHE inhibition produced by amiloride and HMA in hepatoma cells[a]

Cell line	Amiloride[b]	HMA[c]
HTC	65 ± 5	10.5 ± 0.5
HepG2	120 ± 6	20.5 ± 0.5
PLC PRF5	150 ± 7	24.1 ± 2.2

[a] Values were calculated as described in Section 2.5, and are expressed as mean ± S.E. ($n = 3$ different experiments). HMA, hexamethylene amiloride; HTC, rat hepatocarcinoma in tissue culture; NHE, Na^+:H^+ exchange.

[b] Values obtained after 1 h of incubation with amiloride in culture medium.

[c] Values obtained after 5 min of incubation with HMA in culture medium.

In agreement with the above data, reductive metabolism was profoundly affected in the three cell lines. Table 3 shows that MTT reduction decreased significantly after 24 h of exposure to HMA, in the concentration range of the IC_{50} for NHE activity and DNA synthesis inhibition. Lysosomal integrity of HTC and PLC PRF5 cells is as sensitive as the reductive capacity of both cell types. On the other hand, the lysosomal integrity of HepG2 cells appeared to be less sensitive to HMA. In all three cases, mitochondrial viability seemed to be less affected by exposure to HMA than cell viability, probably indicating that mitochondria were not the major target of the cytotoxicity induced by NHE inhibition. Interestingly, plasma membrane integrity, measured by calcein retention, remained almost unaltered by HMA at a concentration of below 25 μM, and even clearly increased in HTC and HepG2 cells (not shown). However, it remained nearly as affected as the two cytotoxicity parameters, MTT reduction and NR retention, in PLC PRF5 cells. Rather than being produced by an increase in intracellular volume or the activation of cytosolic esterase activity, this effect may reflect the features of P-glycoprotein substrates that calcein possesses (Tiberghien and Loor, 1996). At higher concen-

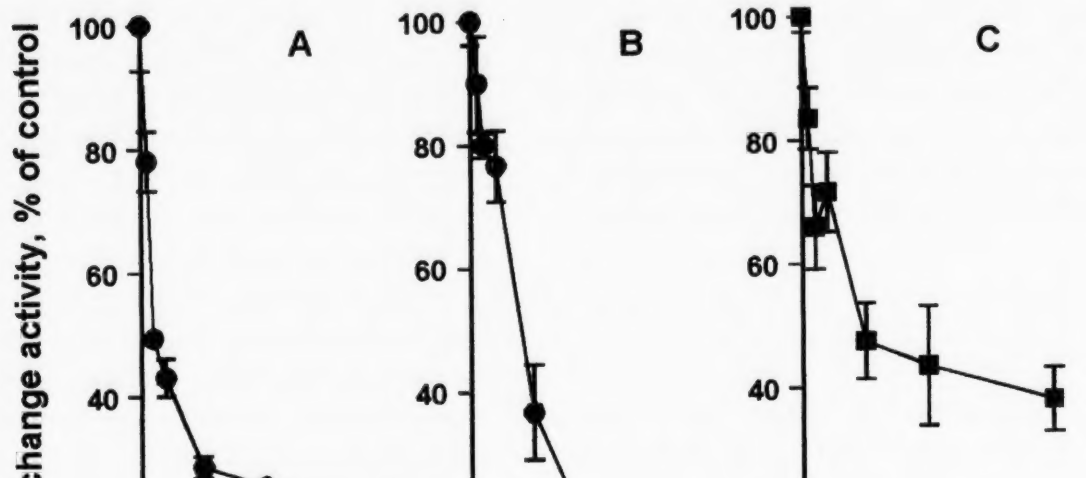

Fig. 1. Titration of hexamethylene amiloride (HMA) on $Na^+:H^+$ exchange (NHE) activity after 5 min of incubation (A–C) and on intracellular pH (pH_i) after exposure for 24 h in complete culture medium (D–F) of rat hepatocarcinoma in tissue culture (HTC) (A, D), HepG2 (B, E) and PLC PRF5 (C, F).

Table 2
Effect of 24-h exposure to amiloride or HMA on cell protein content, DNA, RNA and protein synthesis in tumor liver cells growing logarithmically[a]

Cell line	Cell content		DNA synthesis		RNA synthesis		Protein synthesis	
	Amiloride	HMA	Amiloride	HMA	Amiloride	HMA	Amiloride	HMA
HTC	181 ± 15	18 ± 0.5	64 ± 5	23 ± 3	153 ± 12	36 ± 3	158 ± 13	46 ± 2
HepG2	194 ± 17	22 ± 2	168 ± 15	19 ± 2	464 ± 41	18 ± 1	324 ± 28	38 ± 3
PLC PRF5	47 ± 6	22 ± 2	89 ± 7	10 ± 3	764 ± 65	17 ± 2	47 ± 3	36 ± 3

[a] Numbers represent the IC_{50} (μM) for amiloride and HMA, expressed as the mean ± S.E. ($n = 3$). Values were calculated from extrapolation of the titration data. HMA, hexamethylene amiloride; HTC, rat hepatocarcinoma in tissue culture.

trations, the decrease in plasma membrane permeability was still lower than that encountered in cell viability or lysosomal integrity in HTC and HepG2 cells.

Since lysosomal and mitochondrial damage is, in most cases, the result of increases in intracellular Ca^{2+}, which can induce the formation of free radicals, resulting in lipid peroxidation (Kehrer, 1993), we measured its levels according to the response of the fluorochrome 2′,7′-dichlorofluorescin to a short (4 h) exposure to HMA. As shown in Fig. 2A–C, formation of dichlorofluorescein from the plasma membrane-permeant fluorochrome 2′,7′-dichlorofluorescin-loaded cells increased in a dose-dependent way upon incubation with HMA. It was more intense in PLC PRF5 cells than in HepG2 and HTC cells, in this order. This effect was accompanied by a rather small increase in Ca_i^{2+} in HepG2 (Fig. 2E), which was more pronounced in PLC PRF5 (Fig. 2F) and a slight increase, if any, in HTC cells (Fig. 2D), probably indicating the lack of importance of Ca_i^{2+} increases in the cytotoxic effect of HMA in HTC and HepG2 cells. No increases above 250 nM Ca_i^{2+} were observed at other incubation times between 30 min and 24 h in HepG2 and HTC cells (results not shown). To test the suspicion that HMA-induced intracellular damage involves, at least in part, the above mentioned increases in free radical production, we determined the effect of 5 mM *N*-acetylcysteine, a known antioxidant agent able to maintain reduced intracellular GSH levels, or deferoxamine, a free-radical scavenger agent, on MTT reduction, lysosomal NR and mitochondrial R123 retention, after 4 and 24 h of exposure to 50 μM HMA. Both *N*-acetylcysteine and deferoxamine reversed the inhibition of cell viability induced by HMA, at short exposure times, without having any significant effect on cell viability themselves (less than 10% of control values). This indicates the possibility that an increase in free radical formation induced by NHE inhibition and/or a decrease in free GSH induced by acidification may have been responsible for the decrease in cell viability via lysosomal impairment, as has been observed in other cell types (Czene et al., 1997). However, at longer exposure times, cell damage was partially reversed by *N*-acetylcysteine, but not by deferoxamine, as shown in Tables 4–6 for the three cell lines, respectively.

The effects observed in the presence of *N*-acetylcysteine were confirmed by measuring the

Table 3
IC_{50} of HMA on viability parameters in hepatoma cell lines after an incubation period of 24 h[a]

Cell line	MTT	NR	R123	Calcein
HTC	21.6 ± 0.4	25.7 ± 4.4[b]	56.0 ± 6.7[c]	46.5 ± 8.6[c]
HepG2	11.9 ± 1.9	43.8 ± 6.6[c]	55.9 ± 7.6[c]	44.1 ± 8.3[c]
PLC PRF5	21.2 ± 1.8	23.5 ± 1.4[b]	68 ± 9.7[c]	21.8 ± 1.4[b]

[a] Values are expressed as μM (mean ± S.E., $n = 3$ different experiments). HMA, hexamethylene amiloride; HTC, rat hepatocarcinoma in tissue culture; MTT, 3-(4,5)-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; NR, neutral red; R123, rhodamine 123.

[b] No statistically significant difference with respect to MTT values.

[c] Statistically different, $P < 0.05$.

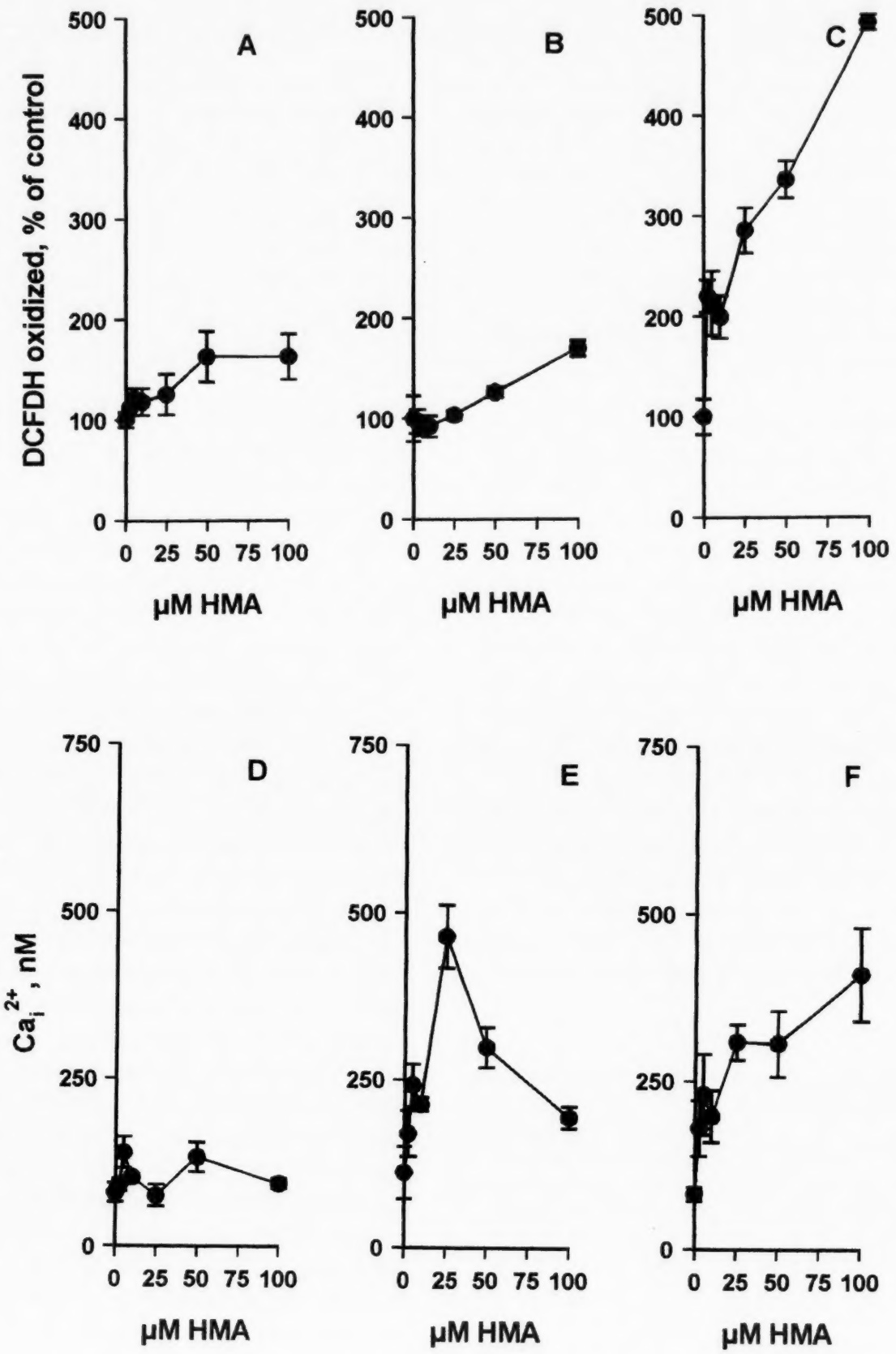

Fig. 2. Free radical production (A–C) and increase in Ca_i^{2+} level (D–F) in rat hepatocarcinoma in tissue culture (HTC) (A, D), HepG2 (B, E) and PLC PRF5 (C, F) in complete culture medium after 4 h in the presence of several concentrations of hexamethylene amiloride (HMA).

GSH levels of cultures treated or not with 50 μM HMA for 24 h. In all the cell lines, HMA decreased the GSH levels (expressed as μg GSH/mg cell protein, mean ± S.E., $n = 6$) with respect to untreated cultures, from 87.3 ± 6.3 to 9.8 ± 1.3 ($P < 0.0001$) for HTC, from 16.7 ± 1.6 to 7.4 ± 0.2 ($P = 0.002$) for HepG2 and from 13.3 ± 1.3 to 7.2 ± 1.5 ($P = 0.03$) for PLC PRF5 cells.

In order to determine whether or not these effects of HMA were directly induced by NHE inhibition and pH_i reduction, we also measured the pH_i levels attained after HMA treatment alone or in combination with NAC or deferoxamine. As shown in Tables 4–6 for the different cell lines, the intracellular acidification promoted by HMA was not reversed by either of the two agents, indicating that pH_i effects on cell viability were a consequence of the NHE inhibition produced by HMA and should occur in the presence of any decrease in GSH levels or any increase in free radical formation.

Taken together, it would seem that amiloride and HMA sensitized tumor cells to quiescence, producing more pronounced cytotoxic or cytostatic effects by inhibiting NHE activity, depending on the level of intracellular acidification attained, the tumor cell line studied, its degree of dedifferentiation and, in particular, the concentration of HMA used.

4. Discussion

It has been observed that to achieve DNA synthesis and growth, tumor cells require increased intracellular Na^+ (Sparks et al., 1983), as well as a faster response to acidic conditions via NHE activity, than normal cells. In this context, the partial inhibition of any of the mechanisms necessary to maintain high intracellular Na^+ concentrations and pH_i, could impair DNA replication and cell growth. Our results clearly indicate that the intracellular acidification produced during exposure to inhibitors of NHE, even in the presence of normal bicarbonate levels in the culture medium, promotes a series of intracellular and metabolic changes in hepatic tumor cells, leading to a reduction in growth activity and, depending on the cell phenotypic characteristics and concentration employed, to loss of cell mass in the cultures. In bone marrow macrophages, there is a clear parallelism among NHE inhibition, DNA synthesis inhibition and accumulation of cells in G_1/S phase of the cell cycle that is mainly ascribed to HMA inhibition of ribonucleotide reductase (Vairo et al., 1992).

Similar effects on cell growth inhibition have been shown in other tumor cell lines of widely different origins, such as leukemia 1210, human gliomas and colorectal carcinomas, where pH_i drops by 0.1–0.2 pH units (Pieri et al., 1983;

Table 4
Reversibility by NAC and deferoxamine of the effect of 50 μM HMA on pH_i, cell viability, lysosomal integrity and mitochondrial viability in HTC cells after 4 and 24 h of incubation[a]

Condition	pH_i	MTT reduction	NR retention	R123 retention
4 h HMA	7.29 ± 0.1	34 ± 1.6	23 ± 2.4	69.3 ± 10
+5 mM NAC	7.25 ± 0.04	79 ± 5[b]	62 ± 7.4[b]	138.7 ± 15.4[b]
+100 μM Def	7.23 ± 0.04	34 ± 3	86 ± 3.4[b]	83.8 ± 7.6[b]
24 h HMA	7.01 ± 0.02	11 ± 5	7.4 ± 4	64.4 ± 4
+5 mM NAC	7.04 ± 0.04	66 ± 3[b]	4 ± 0.2[b]	112.1 ± 12.6[b]
+100 μM Def	7.25 ± 0.04	16 ± 1	6.8 ± 0.3	76.1 ± 4.2[b]

[a] Control pH_i was 7.36 ± 0.08 at 4 h and 7.35 ± 0.09 at 24 h, $n = 8$. Def, deferoxamine; HMA, hexamethylene amiloride; HTC, rat hepatocarcinoma in tissue culture; MTT, 3-(4,5)-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; NAC, *N*-acetylcysteine; NR, neutral red; pH_i: intracellular pH; R123, rhodamine 123.

[b] Significantly different from HMA values, $P < 0.05$. Results are the mean of four separate determinations in three different cultures.

Table 5
Reversibility by NAC and deferoxamine of the effect of 50 μM HMA on pH_i, cell viability, lysosomal integrity and mitochondrial viabiltiy in HepG2 cells after 4 and 24 h of incubation[a]

Condition	pH_i	MTT reduction	NR retention	R123 retention
4 h HMA	7.22 ± 0.02	16 ± 1	21 ± 1.3	54.8 ± 0.5
+5 mM NAC	7.21 ± 0.04	69 ± 1[b]	87 ± 6.2[b]	74 ± 1.7[b]
+100 μM Def	7.20 ± 0.04	35 ± 2[b]	128 ± 15[b]	65.7 ± 0.2[b]
24 h HMA	7.06 ± 0.03	4 ± 0.3	7.5 ± 0.8	48.7 ± 4.2
+5 mM NAC	7.07 ± 0.06	58 ± 1[b]	15.9 ± 1.5[b]	65.1 ± 3.4[b]
+100 μM Def	7.17 ± 0.12	7 ± 0.5[b]	9.4 ± 0.5[b]	40.6 ± 2.6[b]

[a] Control pH_i was 7.26 ± 0.03 at 4 h and 7.26 ± 0.03 at 24 h, $n = 8$. Def, deferoxamine; HMA, hexamethylene amiloride; HTC, rat hepatocarcinoma in tissue culture; MTT, 3-(4,5)-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; NAC, *N*-acetylcysteine; NR, neutral red; pH_i, intracellular pH; R123, rhodamine 123.

[b] Significantly different from HMA values, $P < 0.05$. Results are the mean of four separate determinations in three different cultures.

Szolgay-Daniel et al., 1991). Subtoxic doses of amiloride have been reported to maintain intracellular acidosis in melanoma cells after in vivo glucose administration to tumor-bearing mice (Hedley, 1990). This finding is reflected in our study, in which the degree of acidosis attained in the three cell lines could depend on the glycolytic rate as well as on the mode of glucose utilization to produce lactic acid (Harguindey, 1994). Using murine solid tumors and acid-mediated therapy involving hydralazine, nigericin and HMA, Luo and Tannock (1994) demonstrated the possibility of reducing the tumor surviving fraction in vivo, although, alone, HMA had a weak effect on the particular transplantable tumor used. As far as we know, no such action has been tested yet in vivo using transplantable hepatic tumors, although drug-induced intratumoral acidification seems to have growth retarding effects on several types of tumor in vivo (Wike-Hooley et al., 1984; Newell and Tannock, 1989; Harguindey et al., 1995; Gerweck and Seetharaman, 1996).

Conventional fluorimetric and colorimetric toxicological methods developed in vitro show that HMA provokes cytostatic or cytotoxic effects, depending on its concentration. These effects increase over the course of incubation and are specifically aimed at lysosomes and metabolic activity in the three hepatoma cell lines. We found a substantial impairment in the reductive capacity of tumor cells, paralleled by cell loss, which was closely related to a loss in lysosomal integrity. Surprisingly, however, this was accompanied by an attenuated response of both plasma membrane integrity and loss of mitochondrial membrane polarization, events that indicate specific effects directed at some of these intracellular targets. As has been shown, mitochondrial inner membrane potential impairment is a key point in the development of cell death by apoptosis due to the opening of a permeability transition pore. The latter event is susceptible to inhibition by cyclosporin A at 10 μM or bongkrekic acid, which activates the export of cytochrome *c* into the cytosol and an apoptosis induction factor into the cell nucleus, activating interleukin converting enzyme (ICE)-like caspases and promoting DNA degradation (Kroemer et al., 1998). However, our results indicate a rather small effect on mitochondrial membrane potential, even with long-term incubation periods and rather high doses of HMA, as compared with other effects already shown. Moreover, our unpublished results concerning the impact of cyclosporin A on the effect of HMA on MTT reduction and NR or R123 accumulation indicate no reversion of said effect at 4 or 24 h of incubation with HMA, but rather a deleterious action of cyclosporin A alone or in combination with HMA. This indicates that inhibition of NHE and intracellular acidification induce cytotoxic effects on HCC cells by means other than through impairment of mitochondrial activity alone.

Table 6
Reversibility by NAC and deferoxamine of the effect of 50 μM HMA on pH_i, cell viability, lysosomal integrity and mitochondrial viability in PLC PRF5 cells after 4 and 24 h of incubation[a]

Condition	pH_i	MTT reduction	NR retention	R123 retention
4 h HMA	7.04 ± 0.05	45 ± 8	19 ± 1.3	43.4 ± 2.2
+5 mM NAC	6.92 ± 0.05	129 ± 5[b]	43 ± 3.1[b]	107.4 ± 9.2[b]
+100 μM Def	6.84 ± 0.09	79 ± 11[b]	45 ± 4.4[b]	62.7 ± 11.5[b]
24 h HMA	6.92 ± 0.05	10 ± 0.6	1.1 ± 0.2	73.4 ± 0.2
+5 mM NAC	6.86 ± 0.02	70 ± 9[b]	2.8 ± 0.6[b]	104.5 ± 1[b]
+100 μM Def	6.95 ± 0.01	8 ± 0.6	6.3 ± 3.3[b]	103.2 ± 2[b]

[a] Control pH_i was 7.20 ± 0.03 at 4 h and 7.19 ± 0.05 at 24 h, $n = 8$. Def, deferoxamine; HMA, hexamethylene amiloride; HTC, rat hepatocarcinoma in tissue culture; MTT, 3-(4,5)-dimethylthiazol-2-yl)2,5-diphenyl-tetrazolium bromide; NAC, *N*-acetylcysteine; NR, neutral red; pH_i, intracellular pH; R123, rhodamine 123.

[b] Significantly different from HMA values, $P < 0.05$. Results are the mean of four separate determinations in three different cultures.

The discrepancy observed between the effect of HMA on cell reductive metabolism and mitochondrial integrity could be explained by the fact that MTT reduction is due not only to mitochondrial respiratory chain activity, but also to reduction at the endosomal and lysosomal level, as has been shown for other cell types (Liu et al., 1997). On the other hand, the above effects might be reasonably ascribed to the NHE inhibition produced by HMA or amiloride, which decreases intracellular pH in tumor cells, rather than to side-effects on other metabolic pathways, because of the higher concentrations (in the millimolar range) required to produce them (Kleyman et al., 1988).

The effects on cell death and impairment of viability observed seem to be mediated mainly by the lysosomal dysfunction promoted by a decrease in reductive power, as shown by the restorative effect produced by *N*-acetylcysteine and deferoxamine, as has been pointed out by other authors (Brunk et al., 1997; Hellquist et al., 1997; Nakagawa et al., 1997). This could lead to decompartimentalization of lysosomal enzymes which could provoke cell death. Unpublished work from our laboratory showed that at least amiloride is capable of decreasing intracellular GSH levels and glutathione-*S*-transferase activity in a dose-dependent manner, at least in HTC cells. In this study, we detected clear decreases in GSH levels in the presence of HMA, data that reinforce our present results.

More evidence of the effects promoted by HMA treatment arises from the lack of any substantial effect on mitochondrial impairment in cell death, which does not seem to be accompanied by increases in Ca_i^{2+} in the three cell lines or plasma permeability loss at HMA concentrations able to markedly decrease cell protein content, cell viability and lysosomal integrity. This finding is not controversial if we consider that not all types of cell death require an increase in Ca_i^{2+} (Furlong et al., 1997; Willye, 1997), whereas it has been widely reported that GSH depletion, free-radical production, lysosomal dysfunction and intracellular acidification appear concomitantly with cell death by apoptosis and/or necrosis (Brunk et al., 1997). As our results indicate, a large increase in Ca_i^{2+}, accompanied by the production of free radicals, would probably provoke cell death by necrosis, as seems to be the case of the PLC PRF5 cells, and to apoptosis when the increments were less relevant, as is the case of the HTC and HepG2 cell lines, especially after long incubations in the presence of HMA. Such possibilities are presently being investigated in our laboratory.

Considering the action of amiloride and HMA in calcein retention, that cytosolic rhodamine retention increases in the presence of amiloride (García-Cañero et al., 1998), both being substrates of the multidrug resistance transporter, that its transport alkalinizes intracellularly resistant cells, and that many agents that reverse multidrug resis-

tance induce intracellular acidification (Thiebaut et al., 1990; Hamilton et al., 1993), a role can be proposed for amiloride and its derivatives as modulators of these aspects of paramount interest in liver cancer therapy.

In conclusion, HMA promotes reversible damage to hepatoma cells, independently of its phenotypic characteristics, involving differences in oncoprotein expression, linked to NHE inhibition and the degree of intracellular acidification attained, concurrently with mild, reversible lysosomal damage. In contrast, the damage to hepatoma cells becomes irreversible when lysosomes are deeply impaired. This action seems to be dependent on both the inhibition of cell growth and the reduction of the level of radical scavengers.

Future efforts should focus on the search for other acidifying agents and the potentiation of such effects by the use of more potent NHE inhibitors. In those studies, the use of liver tumor cells in tissue culture, which appear to mimic the clinical situation encountered in HCC, together with the panel of cytotoxicity tests we employed should provide a valuable in vitro model to search for other therapeutic substances sharing this mechanism, which is active against human hepatocarcinomas, as the results of this work point out.

Acknowledgements

We are gratefully indebted to Martha Messman for her editorial help with the manuscript. This work has been supported by DGES/PB-98-0001 from Ministerio de Educacion, Spain.

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Toxicology Letters 106 (1999) 229–235

Toxicology Letters

Cisplatin-induced toxicity in LLC-PK$_1$ kidney epithelial cells: role of basolateral membrane transport

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Received 15 December 1998; accepted 25 March 1999

Abstract

Cytotoxicity of cisplatin was evaluated after apical and/or basolateral treatment of LLC-PK$_1$ cell monolayers grown on porous membrane filters with 300 μM cisplatin. When LLC-PK$_1$ cells were exposed from basolateral side for 0.5–4 h, lactate dehydrogenase (LDH) release into culture medium was markedly stimulated. However, apical treatment of the cells with cisplatin for 0.5 h did not stimulate LDH release. γ-Glutamyltransferase activity and amount of protein in the cell homogenate were markedly decreased by basolateral treatment with cisplatin. However, in the apical treatment with cisplatin, these changes were relatively small, suggesting that degrees of the toxicities were different between basolateral and apical treatment with cisplatin. Cellular platinum level after basolateral treatment with cisplatin was higher compared to that following apical treatment. Furthermore, both accumulation and toxicity of cisplatin in LLC-PK$_1$ cells were decreased by treatment with cisplatin at 4°C. These results suggested that there were specific mechanisms mediating cisplatin uptake at the basolateral membranes of LLC-PK$_1$ cells. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Basolateral membranes; Cisplatin; LLC-PK$_1$ cells; Nephrotoxicity

1. Introduction

Although cisplatin is an effective anticancer agent frequently used in the treatment of solid tumors including those of the testis, ovary, bladder and oropharynx, clinical use of this drug in large doses is limited because of its severe toxic adverse effects, especially nephrotoxicity. It is generally accepted that cisplatin nephrotoxicity is caused by the direct accumulation of cisplatin in the tubular epithelial cells. Cisplatin is secreted via the renal tubules, but precise mechanisms mediating tubular accumulation of cisplatin have not been clarified.

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PII: S0378-4274(99)00071-5

LLC-PK_1 is an established epithelial cell line derived from pig kidney (Hull et al., 1976). The cells form an oriented monolayer with microvilli at their apical side and exhibit characteristics of renal proximal tubular cells, such as transport activity for hexose (Amsler and Cook, 1982; Inui et al., 1984), amino acids (Rabito and Karish, 1983), phosphate (Biber et al., 1983) and organic cations (Inui et al., 1985; Fouda et al., 1990; Saito et al., 1992; Takano et al., 1992). We have been using these cells to study the effects of aminoglycoside antibiotics on cellular function (Hori et al., 1984; Inui et al., 1988; Hori et al., 1993; Takano et al., 1994) and have obtained useful information about aminoglycoside-induced nephrotoxicity. In addition, the mechanisms of cisplatin- (Montine and Borch, 1988) and cyclosporin A- (Scoble et al., 1989) induced nephrotoxicities have been analyzed using LLC-PK_1 cells. When LLC-PK_1 cells are cultured on porous membrane filters, accumulation and/or pharmacological actions of drugs across basolateral and brush-border membranes can be measured separately (Williams, 1989; Saito et al., 1992). In the present study, the toxicity of cisplatin was evaluated by exposing the apical or basolateral membranes of LLC-PK_1 cells to cisplatin independently. We clarified the participation of basolateral membrane transport in cellular accumulation and toxicity of cisplatin.

2. Materials and methods

2.1. Materials

cis-Platinum(II) diammine dichloride (cisplatin) was obtained from Sigma Chemicals (St. Louis, MO). All other chemicals were of the highest purity available.

2.2. Cell culture

LLC-PK_1 cells (ATCC CRL-1392) obtained from the American Type Culture Collection (Manassas, VA) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Life Technologies, Inc., Rockville, MD) with 10% fetal bovine serum (Whittaker Bioproducts Inc., Walkersville, MD), without antibiotics, in an atmosphere of 5% CO_2–95% air at 37°C (Hori et al., 1984; Saito et al., 1992). The cells were subcultured every 4–5 days, using 0.02% EDTA and 0.05% trypsin. For the experiments, cells were seeded on porous membrane filters (3.0 μm pores, 4.71 cm^2 growth area) inside a Transwell® cell culture chamber (Costar, Cambridge, MA) at a density of 5×10^5 cells/cm^2 with complete medium, as described previously (Saito et al., 1992). In the present study, LLC-PK_1 cells were used between passages 218 and 228.

2.3. Experimental design

In the present study, we evaluated the toxicity of cisplatin in LLC-PK_1 cells after exposure of the cells to cisplatin from the apical or basolateral side independently. To evaluate the toxicity of cisplatin, and minimize the diffusion of the drug to the opposite chamber, the following protocol was employed. LLC-PK_1 cells grown on porous membrane filters were exposed to cisplatin from the apical (300–1000 μM) and/or basolateral (50–300 μM) side for specified periods (0.5–4 h). At the end of exposure, the cells were washed, and then incubated for 24 h in fresh medium in the absence of cisplatin to facilitate the alterations of toxicity markers. For measurement of platinum accumulation, LLC-PK_1 cells were treated with 300 μM cisplatin in a buffer composed of 145 mM NaCl, 3 mM KCl, 1 mM CaCl2, 0.5 mM MgCl2, 5 mM D-glucose and 5 mM HEPES (pH 7.4) from either the apical or basolateral side, and then the cells were incubated for the specified periods. At the end of incubation, the cells were washed twice with ice-cold buffer and homogenized in 1.5 ml of distilled water by sonication three times for 5 s each time (VP-5S, Taitec, Saitama, Japan).

2.4. Enzyme assays

For determination of γ-glutamyltransferase (EC 2.3.2.2) and protein amount, LLC-PK_1 cells were washed twice with ice-cold saline, and then homogenized in 1 ml of ice-cold saline by sonication three times for 5 s each time (VP-5S, Taitec). For

determination of lactate dehydrogenase (LDH, EC 1.1.1.27) and γ-glutamyltransferase in culture media, media from apical and basolateral chambers were pooled, and then centrifuged at 3000 rpm for 10 min (CF17D, Hitachi, Tokyo, Japan). LDH activity was determined as described previously (Inui et al., 1981). The release of LDH activity in the culture medium was estimated between approximately 4 and 25% of total activity by a separate experiment. γ-Glutamyltransferase activity was measured as described previously (Hori et al., 1984; Inui et al., 1988). Protein was measured by the method of Bradford (1976), using a Bio-Rad Protein Assay Kit, with bovine γ-globulin as a standard.

2.5. Measurement of inulin permeability across LLC-PK$_1$ cell monolayers

After addition of 10 mg/ml inulin to the basolateral chamber of porous membrane filters, the cells were incubated for specified periods at 37°C. Aliquots of apical medium were kept for determination of inulin concentration. Inulin concentrations in the media were determined according to the method of Dische and Borenfreund (1951) with some modifications.

2.6. Measurement of platinum content in the cell homogenates

Platinum content in the homogenate was determined according to the method of Thompson et al. (1984) with some modifications. The cell homogenates were dried using SpeedVac (Savant Instruments, Inc., New York, NY), and then ashed by incubation with 52% orthophosphoric acid and 0.1 ml of 5% hydrogen peroxide at 70°C for 3 h. After centrifugation at 13,000 rpm for 10 min (CF17D, Hitachi), platinum concentrations in the supernatants were determined by atomic absorption spectrometry (AA670, Shimadzu, Kyoto, Japan).

2.7. Statistical analyses

The data shown in Figs. 1 and 2 were analyzed using two-way ANOVA, after transformation of the data into log base 10. The data from Figs. 3–6 were analyzed by Dunnett's test or Fisher's *t*-test. *P* values of less than 0.05 were considered significant.

3. Results

Fig. 1 shows the time course of the effect of cisplatin on the release of LDH into culture medium. When LLC-PK$_1$ cells were treated with 300 μM cisplatin from the basolateral side for 0.5–4 h, LDH release into culture medium was significantly stimulated compared with that without basolateral treatment ($P < 0.05$). In the apical treatment with cisplatin, LDH release was markedly small compared with that by basolateral treatment during the periods examined. Paracellular permeability was evaluated by measuring inulin flux from the basolateral to the apical chamber simultaneously with cisplatin treatment. Net flux of inulin to the apical chamber was less than 2% of that applied to the basolateral chamber, suggesting that paracellular flux of cisplatin during exposure was negligible.

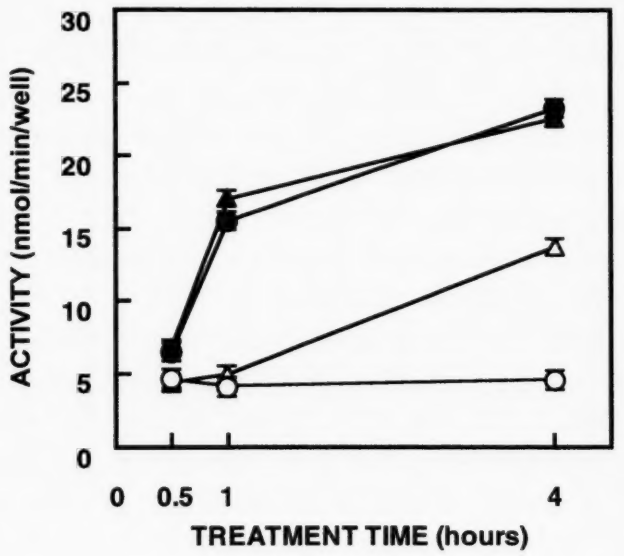

Fig. 1. Effects of apical and/or basolateral treatment with cisplatin on LDH release from LLC-PK$_1$ cells. Six days after seeding, the culture media were replaced with fresh medium (control, ○) or with that containing 300 μM cisplatin (apical, △; basolateral, ●; apical and basolateral, ▲). After 0.5–4 h of treatment at 37°C, the cells were further incubated with fresh culture medium for 24 h, and then LDH activity in the culture medium was determined. Each point represents the mean ± S.E. of three wells.

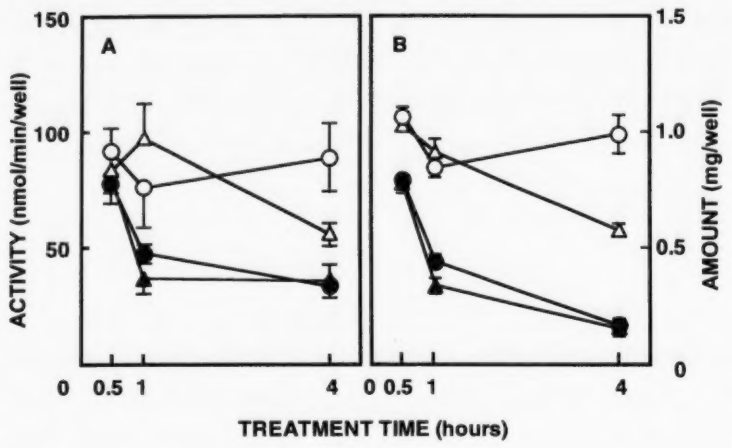

Fig. 2. Effects of apical and/or basolateral treatment with cisplatin on γ-glutamyltransferase (A) and protein content (B) in LLC-PK_1 cells. Six days after seeding, the culture media were replaced with fresh medium (control, ○) or with that containing 300 μM cisplatin (apical, △; basolateral, ●; apical and basolateral, ▲). After 0.5–4 h of treatment at 37°C, the cells were further incubated with fresh culture medium for 24 h, and then γ-glutamyltransferase and protein content in the cell homogenate were determined. Each point represents the mean ± S.E. of three wells. The activity of γ-glutamyltransferase was significantly decreased with basolateral treatment (J, H) at 1 and 4 h, compared to that without basolateral treatment (E, C) ($P < 0.05$). The protein content was significantly decreased by basolateral treatment (J, H) at all time points tested, compared to that without basolateral treatment (E, C) ($P < 0.05$).

Thinning or focal loss of brush-borders was observed by electron microscopy in rats injected with cisplatin (Dobyan et al., 1980). In patients with cisplatin nephrotoxicity, activities of brush-border enzymes, which originated from brush-border membranes of tubular epithelial cells, were detected in urine (Jones et al., 1980). Thus, we measured γ-glutamyltransferase activity in the homogenate of LLC-PK_1 cells treated with 300 μM cisplatin for 0.5–4 h. As shown in Fig. 2A, γ-glutamyltransferase activity in the cell homogenate was markedly decreased by basolateral treatment with cisplatin. Cellular protein content was also decreased by basolateral treatment with cisplatin consistent with the changes in γ-glutamyltransferase activity (Fig. 2B). These results suggested that basolateral uptake of cisplatin plays a more important role than apical uptake in cisplatin-induced toxicity in LLC-PK_1 cells.

Next, the concentration-dependence of cisplatin toxicity was evaluated. LLC-PK_1 cells were treated for 1 h with 50–300 μM or 300–1000 μM cisplatin from the basolateral or apical side, respectively. As shown in Fig. 3, activities of LDH and γ-glutamyltransferase in the medium increased in a dose-dependent manner. The toxicity of basolateral treatment with 300 μM cisplatin was significantly higher than that by apical treatment.

To examine the mechanisms mediating the differences in toxic effects between apical and basolateral treatment with cisplatin, cellular accumulation of platinum was measured after apical or basolateral treatment of LLC-PK_1 cells with cisplatin (Fig. 4). The amounts of platinum after treatment with 300 μM cisplatin for 2 h were 31.8 ± 3.8 and 62.8 ± 5.2 ng/mg protein for apical and basolateral treatment, respectively. These observations suggested that the differences in toxic effects of apical or basolateral exposure to cisplatin were mediated by differential levels of cisplatin accumulation. Therefore, it was assumed that there was a mechanism responsible for cellular accumulation of cisplatin in the basolateral membranes of LLC-PK_1 cells.

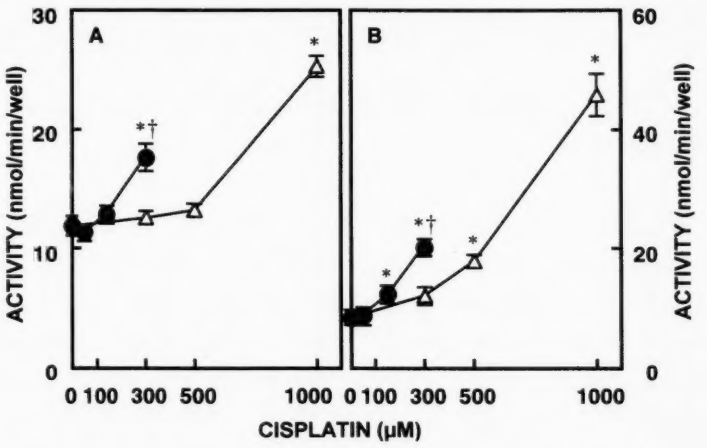

Fig. 3. Effects of various concentrations of cisplatin on the release of LDH (A) and γ-glutamyltransferase (B) from LLC-PK_1 cell monolayers. Six days after seeding, the culture media in apical (△) and basolateral (●) chambers were replaced with fresh media containing various concentrations of cisplatin. After 1 h of treatment at 37°C, the cells were further incubated with fresh culture medium for 24 h, and then LDH (A) and γ-glutamyltransferase (B) activities in the culture media were determined. Each point represents the mean ± S.E. of three wells. *$P < 0.05$, significantly different from control (without cisplatin) by Dunnett's test. †$P < 0.05$, significantly different from apical treatment by Fisher's t-test.

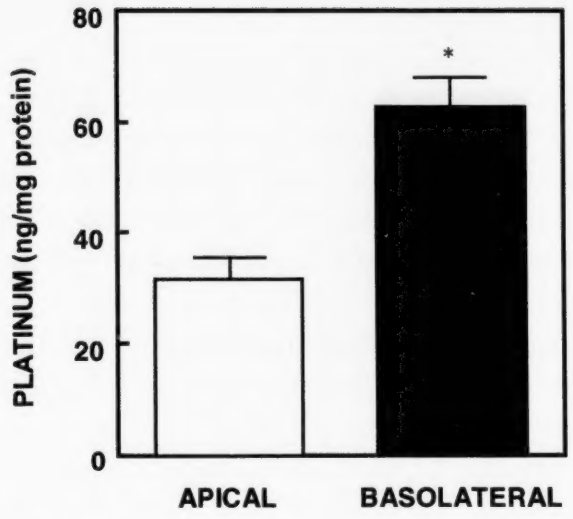

Fig. 4. Platinum contents in LLC-PK_1 cells after apical or basolateral treatment with cisplatin. Six days after seeding, the culture media in apical (open column) and basolateral (closed column) chambers were replaced with buffer or buffer containing 300 μM cisplatin, respectively. After 2 h of incubation at 37°C, the cells were washed and then platinum contents in the cell homogenates were determined. Each point represents the mean ± S.E. of three determinations. $*P < 0.05$, significantly different from apical treatment by Fisher's t-test.

Finally, we examined the effects of temperature on the toxicity and accumulation of cisplatin. After basolateral treatment with 300 μM cisplatin for 2 h at 37°C and 4°C, cellular accumulation of platinum were compared. Cellular platinum level at 4°C was significantly lower than that at 37°C, suggesting that basolateral uptake of cisplatin is sensitive to temperature (Fig. 5). To examine the correlation between accumulation and toxicity of cisplatin, release of LDH and γ-glutamyltransferase following basolateral treatment with cisplatin at 37°C and 4°C was evaluated. As shown in Fig. 6, treatment of the cells with cisplatin at low temperature (4°C) attenuated the release of LDH and γ-glutamyltransferase into the medium, suggesting that temperature-sensitive uptake across basolateral membranes is responsible for the toxic effects of cisplatin.

4. Discussion

In the present study, accumulation of platinum was different between apical and basolateral treatment (Fig. 4). It is possible that two mechanisms were responsible for these differences in cisplatin accumulation. That is, cisplatin may be pumped out at the apical membranes of LLC-PK_1 cells and taken up by a specific mechanism in the basolateral membranes of LLC-PK_1 cells. As shown in Figs. 5 and 6, both toxicity (Fig. 6) and accumulation (Fig. 5) of cisplatin were attenuated by treatment of the cells at low temperature (4°C). If the mechanisms responsible for cisplatin efflux are expressed in the apical membranes, the accumulation of cisplatin should not be decreased, but would rather be increased by low temperature, resulting in increased toxicity. However, treatment with low temperature decreased both accumulation and toxicity of cisplatin, suggesting that specific mechanisms responsible for cisplatin accumulation were present in the basolateral membranes of LLC-PK_1 cells.

Andrews et al. (1988, 1991) reported that cisplatin uptake in ovary cancer cells was decreased by treatment with 2,4-dinitrophenol, sodium fluoride and ouabain. They also reported that the uptake was partially dependent on Na^+ and that Na^+,K^+-adenosine triphosphatase plays a role in

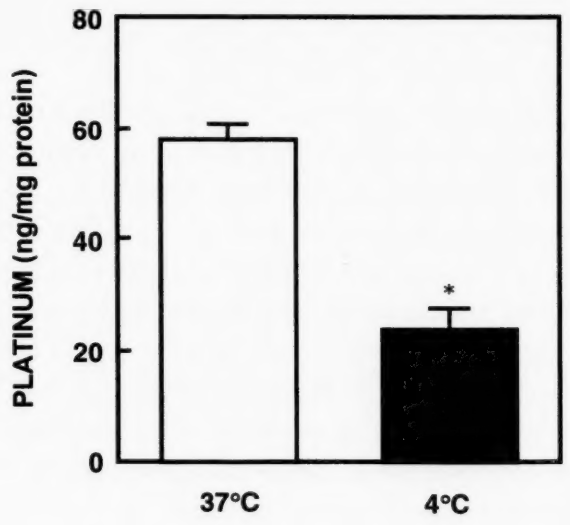

Fig. 5. Effects of temperature on platinum accumulation by basolateral treatment of LLC-PK_1 cells with cisplatin. Six days after seeding, the culture media in apical and basolateral chambers were replaced with buffer or buffer containing 300 μM cisplatin, respectively. After 2 h of incubation at 37°C (open column) or 4°C (closed column), the cells were washed and then platinum contents in the cell homogenates were determined. Each point represents the mean ± S.E. of three determinations. $*P < 0.05$, significantly different from 37°C treatment by Fisher's t-test.

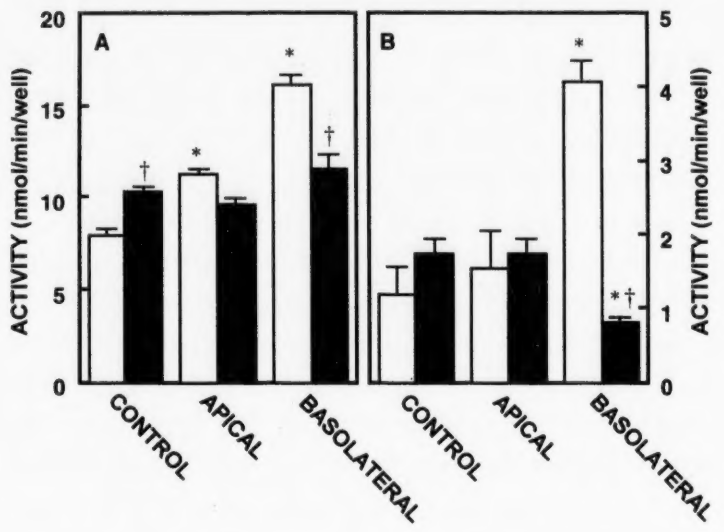

Fig. 6. Effects of temperature on cisplatin-induced release of LDH (A) and γ-glutamyltransferase (B) activities from LLC-PK_1 cells. Six days after seeding, the culture media in apical and basolateral chambers were replaced with buffer or buffer containing 300 μM cisplatin. After 2 h of treatment at 37°C (open column) or 4°C (closed column), the cells were incubated with fresh culture medium for 24 h at 37°C, and then LDH (A) and γ-glutamyltransferase (B) activities in the culture media were determined. Each point represents the mean ± S.E. of three wells. $*P < 0.05$, significantly different from control by Dunnett's test. $\dagger P < 0.05$, significantly different from 37°C treatment by Fisher's *t*-test.

cisplatin uptake by these cells. Safirstein et al. (1984) reported that the uptake of cisplatin by slices of rat kidney cortex was dependent on both energy and temperature, and was inhibited in the presence of tetraethylammonium, torazoline, thiamine and probenecid. In the present study, specific mechanisms for cisplatin uptake were expressed in the basolateral membranes of LLC-PK_1 cells. Thus, it is possible that cisplatin was transported across basolateral membranes of renal tubules, and then caused nephrotoxicity. However, the characteristics of basolateral transport systems in renal tubules and LLC-PK_1 cells must be clarified further.

In conclusion, specific mechanisms responsible for the cellular accumulation and toxicity of cisplatin were expressed in the basolateral membranes of LLC-PK_1 cells. It is possible that such mechanisms mediate the accumulation and nephrotoxicity of cisplatin in the kidney.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan, Grants-in-Aid from the Yamada Science Foundation, and by a grant from the Japan Research Foundation for Clinical Pharmacology.

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Toxicology Letters 106 (1999) 237–246

Toxicology Letters

Immune responses to contact allergens: novel approaches to hazard evaluation

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Received 10 February 1998; received in revised form 25 March 1999; accepted 25 March 1999

Abstract

Progress in our understanding of the immunobiological mechanisms that cause skin sensitization and allergic contact dermatitis has facilitated consideration of alternative approaches to hazard evaluation. One such is the murine local lymph node assay in which, in contrast to more traditional guinea pig tests, sensitizing activity is measured as a function of events associated with the induction, rather than the elicitation, phase of contact hypersensitivity. Activity in the local lymph node assay is dependent upon all of those immunological events that are initiated following first encounter with chemical allergen and which result in the stimulation of T lymphocyte proliferative responses in lymph nodes draining the site of exposure. In this respect the assay embraces in an holistic way the induction of skin sensitization. With the objective of developing in vitro approaches to hazard identification, consideration has been given to discrete immunological responses that characterize the induction of skin sensitization. Most attention has focused upon the changes induced by chemical allergens in the phenotype and function of epidermal Langerhans cells and in cytokine expression. In addition, attempts have been made to identify contact allergens as a function of their ability to provoke in vitro specific responses by unprimed T lymphocytes. These novel approaches to skin sensitization testing and their potential utility in the context of toxicological evaluations are reviewed in this article. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Contact sensitization; Hazard evaluation; Predictive testing

1. Introduction

Allergic contact dermatitis is an important and a common occupational health problem. Many and diverse chemicals have been implicated as causing skin sensitization and there is a need for accurate hazard evaluation. The first approaches to predictive testing were based upon guinea pig methods, some of which have continued to serve toxicologists well (Buehler, 1965; Magnusson and Kligman, 1970). In such assays skin sensitizing activity is determined as a function of cutaneous

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PII: S0378-4274(99)00069-7

hypersensitivity reactions provoked by challenge of previously sensitized animals. The same principle is used in the mouse ear swelling test (MEST), in which challenge-induced increases in ear thickness are measured in sensitized mice (Gad et al., 1986). These methods reflect the basic pathobiology of allergic contact dermatitis, insofar as the disease is characterized by the ability of the inducing chemical allergen to elicit in sensitized individuals dermal inflammatory reactions. Nevertheless, such assays are not without difficulties. For instance, test concentrations at the challenge phase are determined largely by the irritant properties of the chemical and, in the case of guinea pig tests, the end point (challenge-induced erythema) is subjective. As a consequence, there has been a growing interest in the development of alternative methods. New initiatives have been facilitated by a much more sophisticated understanding of the immunobiological processes that result in the acquisition of skin sensitization following topical exposure to a chemical allergen. The way in which an appreciation of the cell and molecular biology of allergic sensitization has been translated into consideration of alternative approaches to hazard evaluation is the subject of this review.

2. Immunobiological mechanisms of skin sensitization

The immunobiology of contact sensitization can be viewed from various perspectives and in varying degrees of detail. At a superficial level it can be seen that the basic tenets of adaptive immunity and allergy apply. Allergic sensitization is induced following topical exposure of a susceptible individual to a local concentration of the chemical allergen which will stimulate an immune response and, in turn, cause skin sensitization. If the now sensitized subject subsequently encounters the inducing allergen on the skin (at the same or a different site) then a more aggressive secondary immune response will be elicited resulting in cutaneous inflammation at the site of exposure and the clinical features of allergic contact dermatitis. Contact allergy is a form of delayed-type hypersensitivity reaction and is dependent upon specific (allergen-specific) T lymphocyte responses and the integrity of cell-mediated immune function. At a more detailed level the way in which topical exposure to a sensitizing chemical translates into the induction of an allergen-specific T lymphocyte response is extremely complex, involving highly orchestrated cell and molecular interactions in the skin and in the lymph nodes draining the site of exposure. It is beyond the scope of this article to consider in detail the panoply of immune processes that together result in sensitization; these have been reviewed extensively elsewhere (Kimber and Dearman, 1996, 1997; Basketter et al., 1999). A brief summary will suffice here, with a more rigorous consideration of those aspects of immune responses to contact allergens that form the basis of proposed novel approaches to predictive testing.

The skin is an immunologically active tissue with the ability to initiate responses following antigenic challenge (Kimber and Cumberbatch, 1992; Kimber, 1996). In the context of contact sensitization, the most important components of the skin immune system are dendritic cells (and particularly epidermal Langerhans cells) and the cytokines that regulate their movement, maturation and function. Langerhans cells (LC) reside in the epidermis where they form a semi-contiguous network that serves as a trap for antigens encountered in the skin. The primary physiological responsibilities of LC are the interaction with, internalization, processing and transport of antigen. The form in which LC interact with chemical allergens is of interest and of relevance. Chemical allergens are haptens and as such are unable to stimulate immune responses until they have associated with macromolecules. Contact sensitizing chemicals are therefore inherently protein reactive, or are metabolized locally in the skin to a protein reactive species (Although no systematic evaluations are available, it is estimated that up to 25% of contact allergens are prohaptens, requiring local metabolic activation.) It is probably haptenated protein that is internalized and processed by LC. (The ability of chemical allergens to associate with proteins provides the basis of one approach to derivation of structure–activity relationships among skin sensitizers—an approach not de-

scribed here.) These antigen-bearing LC are stimulated to leave the skin and to migrate, via afferent lymphatics, to draining lymph nodes. During this migration LC are subject to phenotypic changes which result in their acquisition of immunostimulatory properties and the ability to present antigen to responsive T lymphocytes in the draining lymph nodes. The mobilization of LC and their functional maturation into antigen-presenting dendritic cells (DC) are initiated and regulated by epidermal cytokines, the expression of many of which are induced or enhanced following skin sensitization (Kimber and Cumberbatch, 1992; Kimber et al., 1998, 1999).

The central event in skin sensitization is the stimulation of allergen-specific T lymphocyte responses and this occurs in peripheral skin draining lymph nodes local to the site of exposure and into which antigen-bearing DC have accumulated. Antigen is presented by DC to responsive T lymphocytes which become activated and are induced to divide and differentiate. Cell division results in the selective clonal expansion of allergen-reactive T lymphocytes; an increased number of T cells able to recognize and respond to the inducing chemical allergen representing the cellular basis of skin sensitization. The effective development of sensitization is dependent also upon qualitative aspects of the immune response and the differentiation of appropriate functional subpopulations of allergen-specific T lymphocytes. The two major classes of T lymphocytes, T helper (Th) cells and T cytotoxic (Tc) cells are defined operationally (but not necessarily invariably) by their expression, respectively, of CD4 and CD8 membrane determinants. In both cases functional subpopulations have been defined which differ with respect to their cytokine secretion phenotypes. The major subsets of Th cells are designated Th_1 and Th_2, and of Tc cells, Tc_1 and Tc_2. The current view is that successful sensitization, and the subsequent elicitation of contact hypersensitivity, is dependent upon the availability of Th_1 and Tc_1 cells, each of which are able to produce, among other cytokines, interferon γ (IFN-γ). The picture is complex, however, as there is increasing evidence that the expression of sensitization is subject to a complex interplay between CD4 and CD8 cell subpopulations (Kimber and Dearman, 1998; Basketter et al., 1999).

Recently new approaches to alternative and in vitro test method development have focused upon modelling of one or other of the immunological processes described above that are necessary for sensitization. Before considering some of these it is relevant to mention the murine local lymph node assay (LLNA), a method for the identification of contact allergens which has been developed, evaluated and validated during the last 15 years (Kimber and Basketter, 1992; Kimber et al., 1994; Basketter et al., 1996; Gerberick et al., 1999). The assay is based on consideration of the induction phase of sensitization. The biological event which is measured, and upon which determination of sensitizing activity is based, is the induction of lymph node cell proliferative responses following topical exposure of mice to the test chemical. In this respect the LLNA accommodates an holistic view of the induction phase of sensitization embracing all necessary immunobiological events that are stimulated following topical exposure to a contact allergen and which result in the induction of T lymphocyte proliferation in draining lymph nodes. The question usually being addressed in the context of in vitro method development is whether one or more of the events that characterize the initiation phase of sensitization are selectively associated with exposure to contact allergens. Two main avenues of investigation can be identified: (1) epidermal responses comprising changes induced in LC (or LC-like cells) and/or induced changes in epidermal cell cytokine expression, and (2) the stimulation of T lymphocyte responses. Both of these approaches will be explored.

3. Epidermal cell responses

The epidermis is a rich source of cytokines. Some of these are expressed constitutively, whereas others require an appropriate stimulus for production. Certain cytokines are the products of LC exclusively, others are produced by keratinocytes only and some are expressed by both cell types. An important function of these cytokines is

to orchestrate the activity and movement of LC. Of particular importance in these respects are tumour necrosis factor α (TNF-α), interleukin 1 (IL-1) and granulocyte/macrophage colony-stimulating factor (GM-CSF); the latter cytokine playing an important role in LC maturation. The stimulation of LC migration from the epidermis is dependent upon the availability of TNF-α, an inducible product of keratinocytes, and of IL-1β which in murine epidermis is produced only by LC themselves (Cumberbatch and Kimber, 1992, 1995; Cumberbatch et al., 1997b). For a number of reasons there has been considerable interest in IL-1β and the production of this cytokine by LC. It has been reported that topical exposure of mice to contact allergens, but not to skin irritants, resulted in a very rapid increase in the expression by epidermal LC of IL-1β (Enk and Katz, 1992). This cytokine when administered intradermally to mice induces both the mobilization of LC and their subsequent accumulation as DC in draining lymph nodes (Cumberbatch et al., 1997a) and, as indicated above, IL-1β is a necessary costimulus for the initiation for LC migration during contact sensitization (Cumberbatch et al., 1997b). Investigations in mice have revealed that exogenous IL-1β can effect in the skin many of the changes associated with topical sensitization including, importantly, the production by keratinocytes of TNF-α (Enk et al., 1993). It perhaps comes as no surprise, therefore, that in mice lacking IL-1β contact sensitization is impaired (Shornick et al., 1996). Taken together the available data suggest that IL-1β is an important and essential mediator for the initiation of cutaneous immune responses and that the induced upregulation by contact allergens of the expression by LC of this cytokine may represent a mandatory early step in skin sensitization. It is for this reason that the regulation by chemical allergens of IL-1β mRNA expression by DC has found favour (as yet unsubstantiated by experimental evidence) as a possible basis for an alternative approach to skin sensitization hazard identification (de Silva et al., 1996). The practical difficulty of developing an in vitro model for monitoring changes induced by exposure of DC to chemicals has been, until recently, the unavailability of suitable cells. Within the external tissues and lymphoid organs, DC are only minority populations and it has proven difficult to obtain sufficient numbers of primary cells for certain types of investigation. Moreover, it is well established that DC rapidly display altered phenotypic characteristics following isolation and short term culture (Schuler and Steinman, 1985). The changes associated with culture of LC reflect the maturation of these cells after mobilization and during their migration to draining lymph nodes. The phenotypic changes to which LC are subject confer on them the properties of immunostimulatory DC. At the same time LC lose their ability to internalize and process exogenous antigen (Streilein and Grammer, 1989). An important advance has been the development of methods which permit the expansion of human DC progenitors in culture, using an appropriate cocktail of cytokines, and the ability, again requiring the presence of necessary cytokines, to maintain cells in a state of differentiation similar to that of LC or immature DC (Lenz et al., 1993; Romani et al., 1994; Sallusto and Lanzavecchia, 1994).

Using short term cultures of DC derived from human peripheral blood some investigators have found that certain chemical allergens, but not non-sensitizing skin irritants, are able to provoke an elevated expression of mRNA for IL-1β (compared with a relevant housekeeping gene such as β-actin) (Hoppe et al., 1995; Jonuleit et al., 1995b; Reutter et al., 1997). Although an association between potential allergenicity and elevated IL-1β expression by DC in vitro is, in principle, consistent with what is believed to be one of the earliest cellular responses to skin sensitizers in vivo, the relationship has still to be confirmed. To date only very few contact allergens, and even fewer skin irritants, have been examined and it is by no means certain that cultured DC prepared from all donors will respond in the same way to exogenous chemicals. However, even if there were found to be a close correlation between the ability of a chemical to stimulate increased IL-1β mRNA expression in vitro and its potential to cause contact sensitization, such would not necessarily provide a suitable foundation for an independent predictive test method. In practice hazard identification re-

quires that the chosen method is able to accommodate a wide range of chemicals of diverse structure and of varying allergenic potential. It is true to say that stimulation of IL-1β expression, and indeed many other in vitro methods that have been proposed for skin sensitization testing, have been investigated using comparatively strong, and usually very strong, contact allergens. The challenge will be to determine whether moderate and weak sensitizing chemicals will effect detectable responses in vitro. It can be argued of course that even if only very potent chemical allergens are able, for instance, to influence IL-1β expression in a measurable way, then such an approach would still have some utility as a screening method for the identification of extreme sensitizers. This may be true in theory but it must be recognized that a negative result in such a screening test would necessitate deployment of a secondary analysis using a method known to be of sufficient sensitivity to allow detection of weaker skin sensitizers. It may prove that with an increasing sensitivity of analytical methods it will be possible to detect relevant changes induced in vitro even by skin sensitizers of only relatively modest potency. Alternatively, experience may reveal that our current level of ability to model in vitro the initiation of adaptive immune responses will limit sensitivity to consideration of only those allergens that provoke the most vigorous changes in the skin and in epidermal cells. Time, and further experimentation, will provide an answer. Notwithstanding these considerations, progress has been made in similar or in other areas with the development of new in vitro approaches to sensitization testing.

In the context of epidermal cytokine induction by chemical allergens other opportunities for in vitro methods may exist. For example, it has been proposed recently that it may be possible to identify chemical allergens, and to distinguish allergens from skin irritants, on the basis of induced changes in the production and release by cultured murine keratinocytes of IL-1α, another proinflammatory epidermal cytokine. It was reported that whereas both chemical allergens and skin irritants caused the release by keratinocytes of IL-1α, only the former were associated with an increase in cell-associated IL-1α (Corsini et al., 1998). The authors proposed that these observations could form the basis of an in vitro test method in which skin sensitizers are defined by their ability to enhance levels of keratinocyte-associated IL-1α, where skin irritants are considered to induce only the release of this cytokine and where chemicals are excluded as either allergens or irritants by a failure to induce changes in IL-1α production or release (Corsini et al., 1998). Here again confirmation with a wider range of sensitizing and non-sensitizing chemicals is necessary.

To a large extent, the potential for induced changes in epidermal cytokine expression to provide detailed information on the ability to cause sensitization or dermal irritation has not been explored or exploited fully. In addition to IL-1β, it is known that LC express constitutively, or can be stimulated to express, a variety of cytokines, including some (such as IL-6, IL-12 and IL-18) that are known to play pivotal roles in the initiation and regulation of adaptive immune responses. It may prove that a more detailed appreciation of potential allergenicity is provided by consideration of the kinetics of altered patterns of cytokine expression.

It is apparent that the direct or indirect interaction of chemical allergens with LC or LC-like cells will promote other changes in addition to altered cytokine expression. One such is that topical exposure of mice to skin sensitizing chemicals has been reported to result in the stimulation of absorptive endocytosis by LC. Similar treatment with non-sensitizing chemicals apparently failed to elicit comparable changes and caused only degenerative changes in LC (Kolde and Knop, 1987). There has been considerable interest in the possibility that the endocytic activation of LC may serve as a method for assessing sensitizing activity in vitro. Aiba and Katz (1990) found that exposure of mice to chemical allergens, but not to the skin irritant sodium lauryl sulphate, caused a marked increase in the expression of major histocompatibility complex (MHC) class II (Ia) antigen by a proportion of LC. It has since been proposed that chemical sensitizers may influence not only the level of expression of Ia determinants by LC, but also their distribution (Becker et al., 1992a,b). This latter is assumed to be due to the increased

endocytosis of membrane molecules following encounter of LC with chemical allergens. The functional relevance of this for the processing and subsequent presentation of sensitizing chemicals by LC remains unclear, but the presumption is that the phenomenon of enhanced endocytic activity simply reflects increased membrane turnover as a response by LC to encounter with antigen (Lempertz et al., 1996). Attempts have been made to develop the modification by chemical allergens of receptor-mediated endocytosis into an in vitro method for the assessment of sensitizing activity. Initially light microscopy was used to determine the localization of gold-labelled anti-Ia antibody complexes following exposure of LC-enriched populations of murine epidermal cells to chemical allergens. It was found that sensitizing chemicals caused diffuse intracellular staining which was most pronounced in the submembranous region (Becker et al., 1994). More recently a flow cytometric method based upon differential quenching of fluorescein-labelled anti-MHC class II antibodies has been proposed. The suggestion is that following treatment of human DC with contact allergens fluorescence intensity is preserved due to internalization of antibody–antigen complexes into less acidic compartments compared with untreated cells (Becker et al., 1997).

The same authors have now used cultures of DC derived from human peripheral blood to examine other changes stimulated in vitro by contact allergens, and specifically the induction of tyrosine phosphorylation. It was found that some strong allergens (but not skin irritants) were associated with an increase in phosphotyrosine comparable with that caused by antibody-mediated cross-linking of MHC class II determinants (Kuhn et al., 1998). It is of interest that in the same investigations the protein tyrosine kinase inhibitor genistein was shown to inhibit tyrosine phosphorylation induced by allergens and also the expression of mRNA for IL-1β (Kuhn et al., 1998). A more extensive consideration of the changes induced in LC-like cultured human DC was reported by Aiba et al. (1997). These data are instructive because they point to inter-individual differences in the response of DC to chemical stimulation and also to variations between allergens themselves. The general picture was one of the stimulation by allergens (2,4-dinitrochlorobenzene (DNCB) and nickel chloride) of several changes, including the increased membrane expression of MHC class II molecules, CD54 (intercellular adhesion molecule-1) and CD86 (a costimulatory molecule expressed by antigen presenting cells). An increase in IL-1β production was recorded also. Another potent contact allergen, picryl chloride, was found to provoke responses similar to those seen with DNCB. However, the number of donors whose DC were able to respond to picryl chloride was much smaller than those displaying responses to DNCB or nickel. In addition, DNCB and nickel were found to elicit different patterns of responses with respect to both altered membrane expression of CD54 and MHC class II and induced cytokine production (Aiba et al., 1997). Collectively these data suggest that while certain strong chemical allergens do indeed have the potential to provoke measurable changes in the phenotype of cultured DC, the use of such responses in the context of predictive toxicology will require recognition of the fact that there appear to exist differences between donors (which appear to be both quantitative and qualitative in nature) and between chemical allergens. Moreover, it will be necessary to standardize the use of cultured DC to eliminate as far as possible differences due to variations in phenotype and/or function of target populations. Membrane determinants other than those mentioned above may also be up-regulated (or stabilized) on cultured dendritic cells by exposure to chemical allergens (Jonuleit et al., 1995a), but whether these will prove to be reliable and consistent markers of sensitizing activity remains to be established.

Before leaving epidermal responses it is necessary to consider briefly one additional approach that has been proposed recently. It was reported that treatment of human skin explant cultures with contact allergens was associated with a significant reduction in the frequency of epidermal LC and the accumulation of LC-like cells at the epidermal–dermal junction. In parallel studies skin irritants were found not to affect LC numbers in a similar fashion (Pistoor et al., 1996).

Here again, more extensive investigations with a wider range of chemicals are necessary. In addition, consideration must be given to the practical utility for screening purposes of a method employing human skin explants.

4. T lymphocyte responses

It would appear that in principle an obvious measure of sensitizing activity in vitro would be the ability of chemicals, when delivered in association with appropriate antigen presenting cells, to provoke T lymphocyte proliferative responses. In practice, however, the major difficulty has been the need to obtain a sufficiently strong signal of allergen-induced prolilferation against the background noise of lymphocyte turnover which occurs spontaneously or in response to other stimuli in the culture medium. Another practical problem is that the threshold for antigen-induced activation of potentially responsive naive T lymphocytes is higher than that required for elicitation of responses by T lymphocytes from primed animals. One manifestation of this is that DC appear to be mandatory antigen presenting cells for the stimulation of primary T lymphocyte responses (Inaba and Steinman, 1984). A breakthrough of sorts, which solved both of these problems and which confirmed that primary T cell responses could be induced in vitro, was described by Hauser and Katz (1988). In their investigations hapten-modified LC were used to activate T lymphocytes derived from naive mice. Autoreactive T cells activated by culture with normal (lacking hapten) syngeneic LC were eliminated by treatment with bromodeoxyuridine and light. The residual population of T lymphocytes was shown to be only minimally responsive to culture with normal LC, but to mount a vigorous proliferative response when mixed with hapten-modified LC. In the same series of experiments the specificity of induced primary T lymphocyte responses was considered. Prior removal of T cells responsive to one hapten did not impair restimulation in vitro by LC modified with a second, unrelated chemical allergen (Hauser and Katz, 1988). There have been various attempts to capitalize on these observations, the objective being to provoke allergen-specific T lymphocyte responses using only responder populations from naive mice or from unsensitized humans. For instance, Moulon et al. (1993, 1994) were able to demonstrate that human LC modified in vitro with the hapten trinitrophenyl (TNP; the haptenic moiety deriving from the contact allergen picryl chloride) could provoke primary T lymphocyte proliferative responses.

Recently this model of in vitro stimulation of primary T lymphcoyte proliferative responses has been developed further. It has been reported that responses could be induced to the chemical allergen fluorescein isothiocyanate, as well as to TNP. Moreover, it was confirmed that cultured human LC-like cells modified with these haptens were able to activate $CD45RA^+$ T lymphocytes, this being the phenotypic marker which characterizes virgin T cells. In parallel studies it was found that a skin irritant failed to induce responses (Rougier et al., 1998). Encouraging as these data are it is important to emphasize that in their investigations Rougier et al. (1998) failed to provoke T lymphocyte responses to some other chemical allergens. A similar distinction between strong and weak allergens was found in a previous series of experiments performed by the same authors (Krasteva et al., 1996). An interesting observation deriving from these investigations was that while *p*-phenylenediamine (PPD; a common human skin sensitizer which is in fact a prohapten) failed in most instances to stimulate a T lymphocyte response, Bandrowski's base, a metabolite of PPD, readily induced measurable proliferative activity (Krasteva et al., 1996; Rougier et al., 1998). These data serve to illustrate the importance of considering the need for metabolic activation of prohaptens when evaluating in vitro the potential skin sensitizing activity of chemicals.

Taken together, it would appear that relatively strong contact allergens have the ability to stimulate proliferative responses by naive T lymphocytes when presented in association with LC or LC-like cells derived from peripheral blood progenitors. What remains uncertain is whether the sensitivity of such an approach will be sufficient for the use of allergen-induced T lymphocyte

activation as the basis of a method for routine toxicological assessment of skin sensitizing activity.

5. Conclusions

A better understanding of the immunological processes that are necessary for, and which result in, contact sensitization has created new opportunities for the development of alternative experimental approaches for evaluating allergenic potential. The main issues that need to be addressed if such opportunities are to be translated into realistic in vitro methods for the routine identification of skin sensitizing chemicals are ones of sensitivity and selectivity. To this end it will be necessary to assess potential methods rigorously with non-sensitizing chemicals and with sensitizing chemicals of differing potency. In addition, in all such methods it will be necessary to consider carefully how evaluation of chemicals requiring metabolism to express their sensitizing activity should be approached, and it is possible that a comprehensive in vitro assay will require the incorporation of a metabolic activation step analogous to the Ames test for genotoxic carcinogens. Notwithstanding these considerations, considerable progress has been made and will continue to be made. There is no reason to believe that an in vitro method for screening purposes at least is a realistic goal.

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Toxicology Letters 106 (1999) 247–254

Toxicology Letters

Absence of correlation between telomerase activity and hepatic neoplasia in B6C3F1 mice

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Received 21 January 1999; received in revised form 29 March 1999; accepted 29 March 1999

Abstract

Telomeres are the physical ends of eukaryotic chromosomes, which maintain chromosome stability and are progressively shortened with aging in somatic cells. The enzyme telomerase elongates telometric DNA and while not usually detectable in human somatic cells is expressed in most human tumors. The present study was conducted to determine if telomerase activity is a marker for spontaneous hepatic neoplastic changes in B6C3F1 mice, a strain frequently used in rodent carcinogenicity studies. Telomerase activity was generally higher in microscopically normal liver tissue from 8-week-old compared to aged mice (110-week-old); however, telomerase activity was not consistently increased in hepatocellular adenomas and carcinomas. It is proposed that, while elevated telomerase activity may modulate human tumor development, modulation of telomerase activity is not a feature of hepatic tumors in B6C3F1 mice and therefore is unlikely to have utility as a molecular marker for hepatic neoplasia in this mouse strain. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: B6C3F1 mice; Hepatic tumors; Liver; Telomerase

1. Introduction

The B6C3F1 mouse is the benchmark strain of mice used in rodent carcinogenicity studies. The strain has a background incidence of liver tumors and a propensity for chemically induced liver tumors (Ward et al., 1996). The pathogenesis of murine hepatocellular neoplasia is well described, with foci of cellular alteration progressing to adenoma and carcinoma (Frith and Ward, 1979; Ward, 1980; Maronpot et al., 1987). The morphological features of spontaneous and chemically induced tumors generally do not differ, and therefore molecular markers of initiating events, as well as attempts to differentiate between chemi-

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PII: S0378-4274(99)00073-9

cally induced and spontaneously occurring tumors, have been pursued. The best characterized of these markers are the mutations of the H-*ras* oncogene which occur in approximately half of all spontaneously occurring tumors and in some preneoplastic foci (Ward et al., 1996). The present study was undertaken to determine if changes in another molecular marker, telomerase activity, were associated with malignancy in spontaneously occurring liver tumors in aged B6C3F1 mice.

Telomeres are the physical ends of eukaryotic chromosomes that maintain chromosome stability by preventing enzymatic degradation or illicit recombination events (Blackburn and Greider, 1995). Telomeres are composed of a DNA component and multiple protein components and in most vertebrates telomeric DNA consists of 5′-TTAGGG-3′ sequences repeated hundreds to thousands of times. In humans, telomere length has been implicated in the control of cellular lifespan since telomeric DNA is progressively shortened during cell replication in culture and with aging in somatic cells (Allsopp et al., 1995; Chang and Harley, 1995). It has also recently been shown that telomere elongation in human epithelial cells and fibroblasts leads to extension of lifespan (Bodnar et al., 1998; Vaziri and Benchimol, 1998). Telomerase is an enzyme with an integral RNA component that offsets the loss of telomeric sequences by elongating telomeric DNA through de novo synthesis of the telomeric repeat sequence, effectively compensating for the loss of terminal sequences resulting from replication (Harley et al., 1994; Counter, 1996). In humans, telomerase is generally not detectable in somatic cells, whereas it is expressed in germ cells, bone marrow cells, activated lymphocytes and the vast majority of tumors (reviewed in Shay and Bacchetti, 1997). This has instigated considerable clinical interest in telomerase as a marker for malignancy and as a potential therapeutic target. In mice, telomerase has been detected in some somatic tissues, as well as germ cells. Liver, mammary tissue and spleen have detectable telomerase activity, whereas skin and brain are negative (Prowse et al., 1993; Chadeneau et al., 1995; Prowse and Greider, 1995; Burger et al., 1997; Coviello McLaughlin and Prowse, 1997; Bednarek et al., 1998). However, even in mice, increased telomerase activity has been reported in viral or chemically induced tumors compared to surrounding tissue (Bednarek et al., 1995; Chadeneau et al., 1995; Broccoli et al., 1996; Bednarek et al., 1997). In this study, liver samples for determination of telomerase activity were obtained from spontaneously occurring masses and normal liver tissue in the B6C3F1 mice. Microscopic evaluations of masses and normal tissue were conducted for final diagnosis, which allowed correlation on an individual animal basis.

2. Materials and methods

2.1. Animals/cell samples

Six-week-old, random-bred, barrier-raised male and female B6C3F1 mice (Charles River Canada, St. Constant, Quebec) were quarantined for 2 weeks and clinically healthy animals housed individually for an additional 104 weeks. The animal room temperature was maintained at 22 ± 4°C at a relative humidity of 30–70%. Animal housing and care were in compliance with the Animals for Research Act of Ontario, 1980, and as amended in 1989, and within the guidelines of the Canadian Council on Animal Care. The animals were euthanized by CO_2 inhalation and necropsied. Samples of approximately 100 mg of skin and liver were collected for determination of telomerase activity from one group of 13 male and 11 female 2-year-old mice and one group of ten male and ten female 8-week-old mice. Two samples of liver were collected from 2-year-old mice with liver masses: one sample of grossly normal liver and one sample from the mass. All liver samples for determination of telomerase activity obtained from masses and grossly normal tissue were also evaluated microscopically. The tissue samples were flash-frozen in cryotubes in liquid nitrogen or dry ice and ethanol and stored at -70°C until analysis.

Linearity of the telomerase assay was conducted using chronic myelogenous human leukemic K-562 cells (American Type Culture Collection, Rockville, MD). The cells were cul-

tured at 37°C, 5% CO_2, 90% humidity at concentration of 10^5 cells/ml in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin and harvested for determination of telomerase activity at exponential growth phase.

2.2. Histopathology of the liver

Liver was fixed in 10% cold neutral buffered formalin for 24 h, processed and embedded in paraffin. Sections were stained with hematoxylin and eosin and evaluated microscopically for the presence of tumors and other lesions. Diagnostic criteria for hepatic tumors were based on published literature (Frith and Ward, 1979; Ward, 1980; Maronpot et al., 1987) and are summarized in Table 1.

2.3. Tissue preparation

The tissues were weighed, minced and lysed with 2 μl of 1 × CHAPS lysis buffer (Oncor Inc., Gaitherburg, MD), per mg tissue, and RNase inhibitor (100 U/ml) (Canadian Life Technologies Inc., Burlington, Ontario) was added before homogenization on ice. K-562 cells were washed twice with PBS and then resuspended with 1 × CHAPS lysis buffer (1 μl per 10^4 cells and 100 U/ml RNase inhibitor added). Samples were incubated on ice for 30 min, centrifuged at 12,000 × *g* for 20 min at 4°C and the supernatant harvested. The supernatants were either frozen at −70°C or assayed immediately and an aliquot was used for protein determination using a BioRad DC protein determination kit (BioRad Laboratories Inc., Mississauga, ON).

2.4. Telomerase activity

Telomerase activity was determined in a two-enzyme system according to Kim et al. (1994) and Piatyszek et al. (1995), with modifications as described in the TRAPeze Telomerase Detection Kit (Oncor Inc., Gaithersburg, MD). Briefly, substrate oligonucleotide primer (TS primer) and DNA molecular marker were end-labeled with [γ-^{32}P]ATP (Amersham Canada Ltd, Oakville, Ontario) at 37°C for 20 min and then at 85°C for 5 min. The samples were incubated with labeled TS primer, nucleotides and Taq polymerase at 30°C for 30 min with telomerase adding 6 base-pair (bp) telomeric repeats to the 3′-end of the labeled TS primer. Reverse primer (RP primer) and a template for amplification of a 36-bp internal standard were included in the reaction mixture. The telomerase extension products and the internal standard were then amplified by polymerase chain reaction (PCR) using the TS and RP primers. The incubation mixture was immediately transferred to a thermal cycler (Perkin-Elmer GeneAmp PCR system 2400), prewarmed to 94°C for 30 cycles of a two-step program: 94°C for 30 s, 60°C for 30 s, then held at 72°C for 5 min. A

Table 1
Diagnostic criteria for hepatocellular adenoma and carcinoma

Hepatocellular adenoma
Discrete, well-delineated nodule of variable size
Compression of adjacent tissue
Uneven growth patterns
No evidence of metastasis or invasion of adjacent parenchyma
Cells may form irregular cords or be arranged in a solid pattern
Neoplastic hepatocytes arranged perpendicular to normal hepatic cords at periphery of tumor
Well-differentiated cells; may be larger or smaller than normal hepatocytes
Relatively uniform population
Little or no nuclear pleomorphism
Low mitotic index
Absence of portal triads or normal hepatic lobular architecture
Hepatocellular carcinoma
Distinct trabecular, solid or adenoid pattern, or haphazardly arranged cells
Plates of cells generally more than one cell layer thick and irregular
Locally invasive and may metastasize to the lungs
Often contain wide irregular vascular channels
May contain areas of hemorrhage and/or necrosis
Cellular pleomorphism common, cells vary from well-differentiated to anaplastic; cells may be eosinophilic or basophilic
Variable nuclear size, increased nuclear to cytoplasmic ratio
Mitotic index usually high; bizarre mitotic figures occasionally seen

25-μl aliquot of each sample was then electrophoresed on a 12.5% ND-polyacrylamide gel in 0.5 × TBE at 250 V for approximately 2 h. The resulting gel was mounted on filter paper, wrapped in plastic and placed in a phosphor storage cassette overnight. A 16-bit grey level image was acquired using a Molecular Dynamics PhosphorImager SI (Model 7) and the area and intensity of the bands determined using Molecular Dynamics ImageQuant software (Version 4.2). Each sample was analyzed in duplicate and a heat-inactivated test extract included for all samples. The intensity of the bands corresponding to the telomerase product ladder was determined in non-heat-treated and heat-treated tissue extract and the telomerase activity expressed as total product generated (TPG). The difference in intensity of the ladder between non-heat-treated and heat-treated samples divided by the intensity of 36-bp internal standard in the heat-treated lane was used as a measure of the telomerase activity in the sample. TPG was obtained by normalizing this value with the ratio obtained using a Oncor-supplied quantity control run on each separate gel.

2.5. Statistical analysis

Data were analyzed by one-factor analyses of variance at a 5% significance level.

3. Results

3.1. Liver histopathology

There were no histopathological lesions in liver from 8-week-old mice. In the group maintained for 2 years, neoplastic changes (hepatocellular adenoma, hepatocellular carcinoma and hepatocholangiocellular adenoma) were observed in eight males and two females. Samples of liver without neoplastic changes were obtained in nine out of these same ten animals. Three of the males had no histopathological changes in these sections, whereas in the remaining four males and the two females various non-neoplastic changes were observed, including foci of hepatocellular alteration, multifocal infiltrates of mixed inflammatory cells and extramedullary hematopoiesis. In 2-year-old animals without neoplastic changes, there were no histopathological changes in liver from the five males, while in the nine females foci of hepatocellular alteration, multifocal mixed inflammatory cell infiltrates and extramedullary hematopoiesis were observed.

3.2. Telomerase activity

A representative example of PCR-amplified telomerase products is illustrated in Fig. 1. Telomerase activity was detectable in all liver samples from 8-week-old mice and 31 of 35 samples from 2-year-old mice. Telomerase was not detectable in four liver samples from 2-year-old animals or in any of the skin samples from 8-week and 2-year-old mice. The intensity of telomerase amplified products in liver samples was within the intensity range found to be linear using the K-562 cells. The intensity of the bands of telomerase products increased linearly with the log of the number of K-562 cells assayed. Using a fixed number of K-562 cells, the PCR amplification of telomerase products was also shown to increase exponentially between 28 and 40 cycles. Therefore, 30 cycles were used for all assays. Liver samples did not contain Taq inhibitors since the 36-bp internal standard was amplified in all samples. In addition, when extracts from selected samples were mixed with the positive control and assayed with the control there was no indication of a reduction in telomerase activity.

Telomerase activity was not consistently increased in samples of hepatocellular adenomas or carcinomas (Fig. 2) compared to microscopically normal samples or samples with non-neoplastic changes. Of the animals with sections of both neoplastic and non-neoplastic or no histopathological changes, telomerase activity varied considerably between the liver samples. However, telomerase activity was not consistently higher in tumors than in non-tumorigenic tissue. In some animals, samples adjacent to neoplastic changes had lower activity than samples from regions without histopathological or non-neoplastic changes, while in others the opposite pattern was

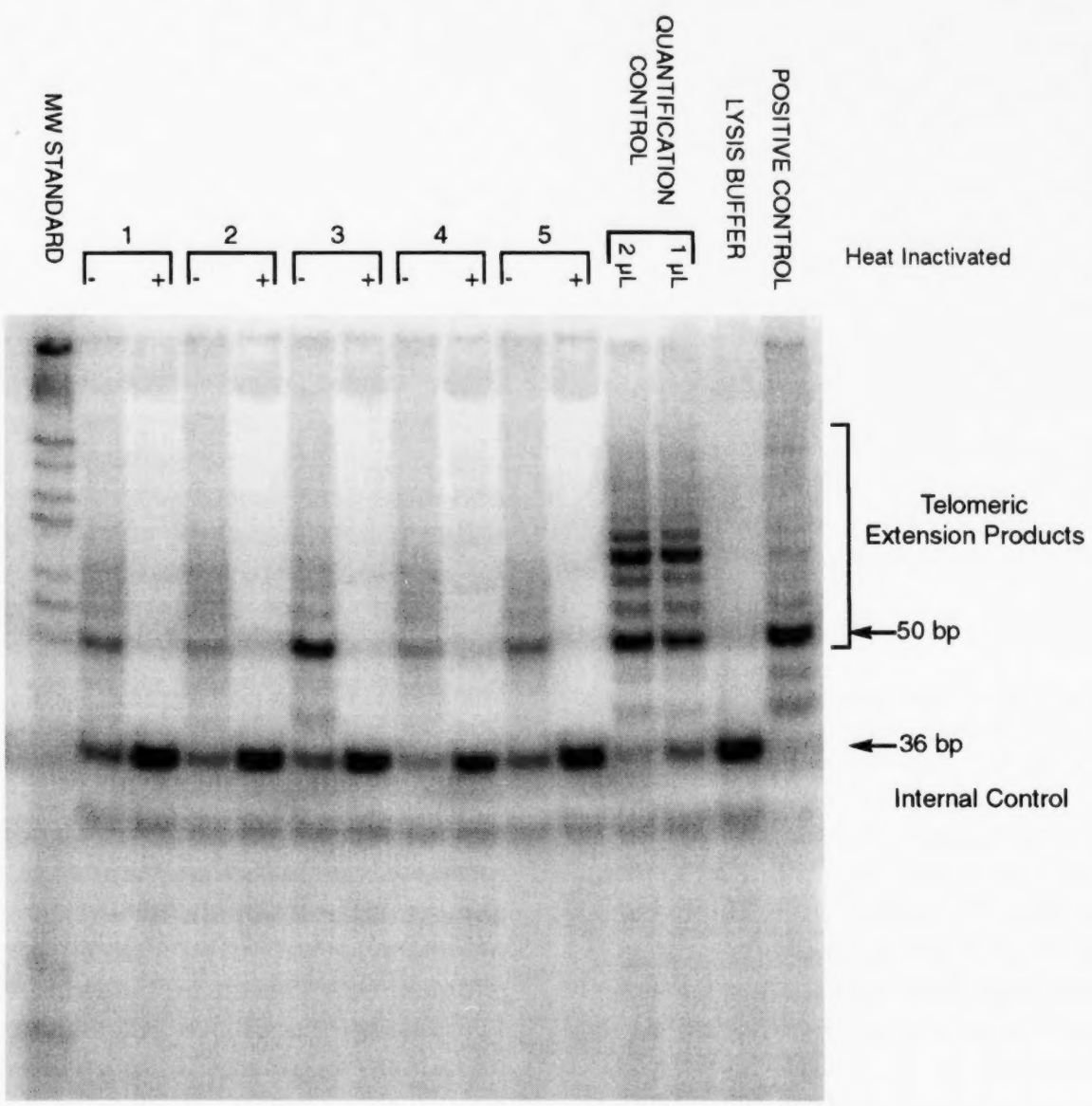

Fig. 1. Telomerase PCR extension products assayed using the TRAP assay in samples from liver from 8-week-old male mice, 2 µg protein per sample were assayed. Samples 1–5 are from individual animals and alternating lanes of liver tissue were conducted on aliquots that were heat-inactivated (+ 85°C, 10 min) prior to incubation for telomerase activity.

observed. Telomerase activity was more uniform in liver tissue that was microscopically normal, while in 2-year-old mice a great deal of variability was observed in samples with neoplastic and non-neoplastic changes. In the young age group, telomerase activity was higher in females than in males, whereas there were no apparent gender differences in the older animals.

4. Discussion

This study showed that telomerase activity was not consistently increased in hepatocellular adenoma and carcinoma compared to surrounding liver tissue in B6C3F1 mice. In humans, telomerase activity is not detectable in normal liver tissue. Low to moderate telomerase activities are detected in large nodules in cirrhotic livers, with telomerase activity generally increasing as hepatocellular carcinoma progresses from well to moderately to poorly differentiated. It has therefore been proposed that telomerase activation is an early event in human hepatocellular proliferation and may facilitate the action of other factors in the process of carcinogenesis to hepatocellular carcinoma (Kojima et al., 1997; Nakashio et al., 1997; Hytiroglou et al., 1998). Our results suggest

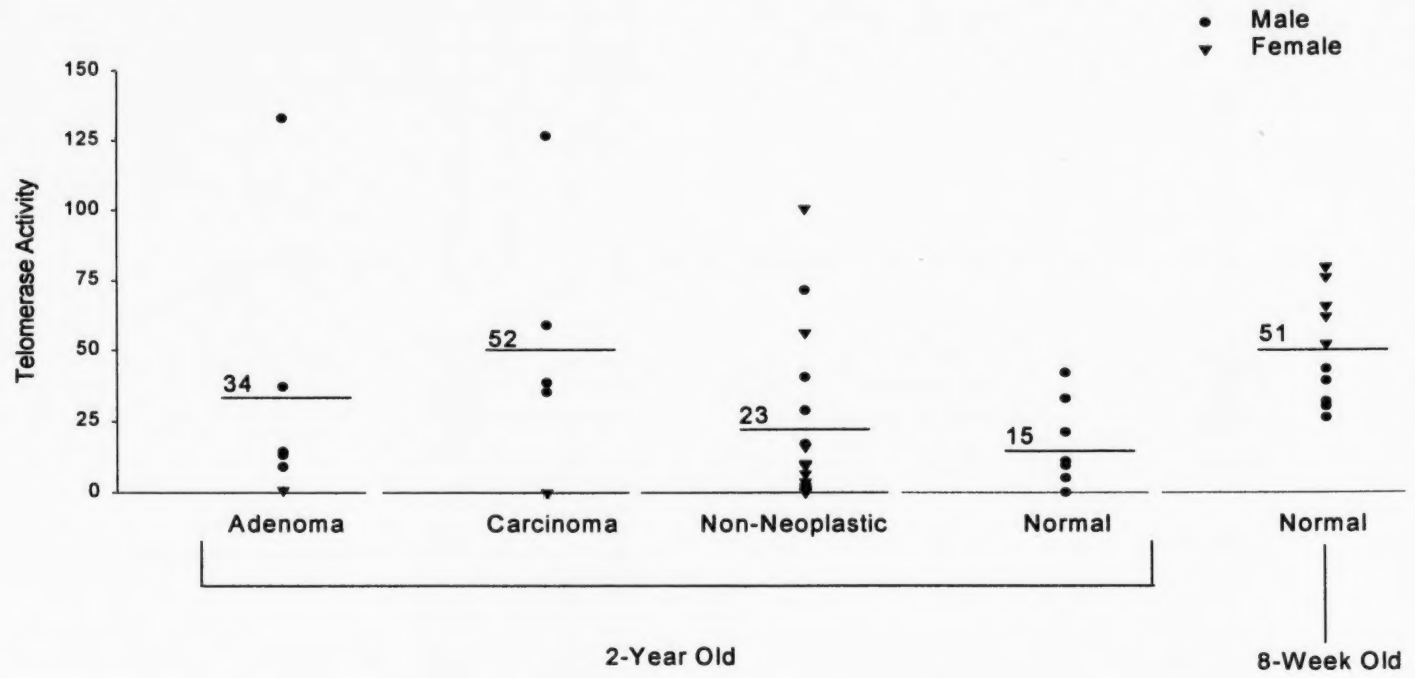

Fig. 2. Telomerase activity (total product generated) in mouse liver with indicated histological diagnoses. Non-neoplastic changes included foci of hepatocellular alteration, multifocal mixed inflammatory cell infiltrates and extramedullary hematopoiesis. The average values for the four categories are indicated with the horizontal bars.

that the role of telomerase in liver tumor formation in B6C3F1 mice may be different than in humans. The B6C3F1 mouse has a high background incidence of liver tumors with incidences of hepatocellular adenoma and carcinoma in untreated control males averaging 30% and in females 8% (Maronpot et al., 1987). Liver is also the most common site for treatment-related tumor development in long-term rodent carcinogenicity studies (Ward et al., 1996). The relevance of this animal model for evaluation of safety of chemicals in humans has been debated and our study suggests that factors involved in mouse liver carcinogenesis are different than in humans.

In tumor development, cellular immortalization is viewed as an obligatory step and it has been argued that the greater ease with which cellular immortalization occurs in rodents, compared to humans, render rodents inherently more susceptible to chemically induced tumors (Newbold et al., 1993). It has been proposed that, when somatic cells divide in the absence of telomerase, the erosion of telomeres limits the number of mitosis that a cell can undergo before exposing the uncapped chromosome ends which would signal the cell to become arrested. Activation of telomerase would maintain the chromosome caps and allow the cell to divide indefinitely and thus potentially become immortal (Harley et al., 1994). The proposed roles of telomere length in cellular senescence and telomerase activation in immortalization have led to the suggestion that the lower tumor incidence in humans compared to mice may, in part, be related to a more stringent control of cellular lifespan in humans by the telomeres/telomerase system (Kipling, 1997; Greider, 1998). Telomerase is expressed in some somatic tissues of the mouse and the telomere length of 20–150 kilobase (kb) in inbred strains of *Mus musculus* greatly exceeds the 15–25-kb telomere length in human germ cells (Kipling, 1997). In models for multistage carcinogenesis in the mouse, telomerase activation or increased telomerase activity has been shown. In chemically induced papillomas in the mouse, there was a progressive increase in telomerase activity with an increased level of genomic instability (Bednarek et al., 1995, 1997). Telomerase activity was generally elevated in primary mammary tumors compared to adjacent tissues in transgenic mice (Chadeneau et al., 1995; Broccoli et al., 1996). There is, however, increasing evidence suggesting that the control of telomere length and

telomerase expression with respect to cellular immortalization in the mouse may be different than in human (Kipling, 1997). Mice with stable genomic deletion of the RNA component of telomerase (mTR −/−) were viable and fertile for six generations and cells from mTR −/− mice were, in the absence of telomerase, able to immortalize in culture and upon transfection became tumorigenic in nude mice (Taylor et al., 1996; Blasco et al., 1997; Lee et al., 1998) The results of the present study suggest that the most common spontaneously occurring tumor type in B6C3F1 mice is not associated with increased telomerase activity. Indeed, no telomerase activity was detected in one adenoma and one carcinoma, and some well-differentiated adenomas had higher telomerase activities than carcinomas.

The regulatory control of telomere length and telomerase activity has not been fully characterized and appears to be complex. The expression of mRNA for mTR does not correlate with telomerase activity in isolated tissue (Blasco et al., 1996) and control of the telomere length has been mapped to a locus on chromosome 2 distinct from candidate genes encoding for telomerase binding proteins and telomerase components (Zhu et al., 1998). Telomere length and telomerase expression is tissue specific and appears to be under developmental control in mice (Prowse and Greider, 1995; Coviello McLaughlin and Prowse, 1997). Gender differences in telomere length have also been demonstrated, with females having significantly longer telomeres than males (Coviello McLaughlin and Prowse, 1997). In the present study, telomerase activity in females was higher than in the males at 8 weeks, which may indicate hormonal influences in control of telomerase activity.

In conclusion, this study suggests that modulation of telomerase activity is not a feature of hepatic tumors in B6C3F1 mice, and that telomerase activity may not be a useful marker for malignancy.

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Toxicology Letters

June 1st 1999

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